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(54) Title: HYDROGEL FILMS AND METHODS OF MAKING AND USING THEREFOR

(57) Abstract: The present invention provides improved hydrogel films useful for therapeutic treatments. The invention also provides materials and methods for modification and polymerization of polysaccharides into hydrogel films, which swell after exposure to a neutral aqueous solution. The methods may include modification of a polysaccharide having at least one carboxylic acid group into a polysaccharide dihydrazide derivative, which is then crosslinked with a polyaldehyde to create a hydrogel film. The invention also relates pharmaceutical compositions composed of a pharmaceutically-acceptable compound and a hydrogel film of the invention. The invention also relates to using the hydrogel films and pharmaceutical compositions of the invention.

HYDROGEL FILMS AND METHODS OF MAKING AND USING THEREFOR

I. ACKNOWLEDGEMENTS

This invention was made with government support under Grant N0:1R01DC04336 awarded by the NIH. The government has certain rights in the invention.

II. CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority to United States Provisional Application No. 60/218,725 filed July 17, 2000 entitled "Hydrogel Films and Methods of Use Therefor," which application is hereby incorporated by this reference in its entirety.

III. FIELD OF THE INVENTION

This invention relates generally to hydrogel films and methods of making and using them.

IV. BACKGROUND OF THE INVENTION

Glycosaminoglycans (GAGs) are a class of biocompatible polymers. For example, hyaluronic acid (HA), which is a member of the GAG family, is a naturally-occurring biopolymer composed of repeating disaccharide units of N-acetyl-D-glucosamine (GlcNAc) D-glucuronic acid (GlcUA) found in the extracellular matrix of all higher animals. Hyaluronic acid (HA) is a GAG. For a discussions of HA and GAGs please see for example Laurent *et al.*, 18 *Acta Chem Scand* 274 (1964), Yui *et al.*, 22 *J. Controlled Rel.* 105 (1992), Tomihata and

Ikada, 18 *Biomaterials* 189 (1997), Shah and Barnett, 480 *ACS Symposium Series* 116 (1991), Larsen *et al.*, In *Cosmetic and Pharmaceutical Applications of Polymers* C.G. Gebelein, Ed.; Plenum Press: New York, 147 (1991), Kuo *et al.*, 2 *Bioconjugates Chem* 232 (1991), Pouyani *et al.*, 116 *J Am Chem Soc* 7515 (1994), Vercruysse *et al.*, 8 *Bioconjugate Chem* 686 (1997), U.S. Patent Numbers 4,582,865, 4,713,448, 5,616, 568, 5, 652,347, and 5,874,417, European Patent Application 0216453 which are herein incorporated by reference at least for their material related to GAGs and HA.

Hydrogel films have received attention as drug delivery vehicles due to their compatibility with most tissues and ability to manipulate their permeability in response to different solutes. All of the existing technologies present difficulties, however, because the alkaline conditions or high temperatures necessary to create hydrogel films with high mechanical strength are cumbersome and harsh. Additionally, many reactions employ an excess of small molecular crosslinking reagents which then require considerable purification in order to obtain material suitable for physiological use. The present invention addresses these problems.

HA forms highly viscous aqueous solutions, and it takes on an expanded random coil structure due to strong hydrogen bonding. The coiled structure allows it to trap approximately 1000 times its weight in water. HA has unique physiochemical properties as well as distinctive biological functions. These functions, relationships, and interactions are discussed for example in Laurent, T. C., Laurent, U. B. G., and Fraser, J. R. E. (1995) Functions of hyaluronan. Ann Rheum Dis 54, 429-432; Fraser, J. R. E., Laurent, T. C., and Laurent, U. B. G. (1997) Hyaluronan: Its nature, distribution, functions and turnover. J Intern Med 242, 27-33; Dowthwaite, G. P., Edwards, J. C. W., and Pitsillides, A. A. (1998) An essential role for the interaction between hyaluronan and hyaluronan binding proteins during joint development. J Histochem Cytochem 46, 641-651; Collis, L., Hall, C., Lange, L., Ziebell, M. R., Prestwich, G. D., and Turley, E. A. (1998) Rapid hyaluronan

uptake is associated with enhanced motility: implications for an intracellular mode of action. FEBS Lett. 440, 444-449; Hardwick, C., Hoare, K., Owens, R., Hohn, H. P., Hook, M., Moore, D., Cripps, V., Austen, L., Nance, D. M., and Turley, E. A. (1992) Molecular cloning of a novel hyaluronan receptor that mediates tumor cell motility. J. Cell Biol. 117, 1343-1350; Entwistle, J., Hall, C. L., and Turley, E. A. (1996) Receptors: regulators of signalling to the cytoskeleton. J Cell Biochem 61, 569-577; and Cheung, W. F., Cruz, T. F., and Turley, E. A. (1999) Receptor for hyaluronan-mediated motility (RHAMM), a hyaladherin that regulates cell responses to growth factors. Biochem. Soc. Trans. 27, 135-142; Toole, B. P. (1997) Hyaluronan in morphogenesis. J Intern Med 242, 35-40; and Kim *et al.*, 9 *Pharm Res* 283, (1992), which are herein incorporated by reference at least for their material related to GAGs and HA and their function and properties.

Another member of the GAG family is chondroitin sulfate (CS). CS is comprised of alternating units of β-1,3-linked glucuronic acid and (β-1,4) N-acetyl-galactosamine (GalNAc) and is sulfated on the 4- or 6- position of the GalNAc residues. CS is usually found bound to a core protein forming a proteoglycan, e.g. aggrecan or versican. Aggregan is the primary proteoglycan in cartilage, and its primary function is to swell and hydrate the collagen fibril framework. Versican is believed to play a role in intracellular signaling, cell recognition, and connecting ECM components to cell surface glycoproteins. Additionally, CS proteoglycans like neurocan and phosphacan play important roles in axon growth and pathfinding.

Wound healing is a complex and orderly sequence of events that involves a variety of cell-types and subcellular signals. Prompt wound repair is vital for patient recovery. It has been thought that GAG and HA may have a role in wound repair (Kearney, J. Wound healing. In Principles and Practice of Burns Management; J. A. D. Settle, Ed.; Churchill Livingstone: New York, 1996; pp 187-195 and Margolis, R. U., and Margolis, R. K. (1997) Chondroitin sulfate proteoglycans as mediators of axon growth and pathfinding. Cell Tissue Res 290, 343-348; Clark, R. A. F. Wound

repair: Overview and general considerations. In The Molecular and Cellular Biology of Wound Repair; R. A. F. Clark, Ed.; Plenum Press: New York, 1996; pp 3-50; Iocono, J. A., Krummel, T. M., Keefer, K. A., Allison, G. M., and Paul, H. (1998) Repeated additions of hyaluronan alters granulation tissue deposition in sponge implants in mice. Wound Repair and Regeneration 6, 442-448; and Sorrell, J. M., Carrino, D. A., Baber, M. A., and Caplan, A. I. (1999) Versican in human fetal skin development. Anat. Embryol. 45-56. which are herein incorporated by reference at least for their material related to wound healing and GAG and HA.)

Severe burn injuries can cause extensive full-thickness skin loss and are accompanied by immunosuppression, which contributes to more than 10,000 fatalities and 100,000 hospitalizations each year. The survival rates for patients with burn injuries have improved dramatically in the past two decades. The cited reasons for this improvement are better understanding of the resuscitation process, improved antibiotics (both systematic and topical), improved nutritional support for the hypermetabolic-catabolic effects of burn injuries, and perhaps most importantly, the recognition that early excision of devitalized tissue promotes closure of the burn wound and is the most effective method of preventing sepsis and multiple organ system failure. Despite these advantages, the mortality rate for patients suffering from burns covering greater than 70% body surface area remains high.

Effective treatment of these injuries would be to ultimately replace the damaged skin by wound closure with stable tissue while restoring normal immune function. At present, autografting remains the most effective method of obtaining suitable permanent wound coverage in thermal injury. Although effective, skin graft donor sites are extremely painful procedures. Burn patients often state that donor sites are more painful than the graft, because functioning nerve endings are left exposed. Further, donor sites are one of the largest factors associated with burn-injury recovery. Often donor sites need to be harvested multiple times to sufficiently cover the original burn site. Time to recovery is dependent upon the re-

epithelialization and healing of the donor sites. Therefore, decreasing the time associated with re-epithelialization and healing of the donor sites will minimize the extended pain and discomfort associated with the practice. Additionally, expediting the healing of each donor site will allow for rapid re-harvesting, allowing the patient to return to normal life activities more quickly.

Prevention of postsurgical adhesion: Intergel (FeHA, formerly Lubricoat) which is a formulation of HA electrostatically crosslinked with trivalent iron, Hylagel which is an engineered hylan gel or membrane device, and Seprafilm which is prepared by blending two anionic polymers, HA and carboxymethylcellulose (CMC) have been developed as a physical barrier for prevention of post-surgical adhesions. (see for example, diZerega GS. Development and clinical evaluation of INTERGEL adhesion prevention solutions for the reduction of adhesions following peritoneal cavity surgery. In: New Frontiers in Medical Sciences: Redefining Hyaluronan. Abbazia di Praglia, Padua, Italy: 1999 and Burns JW, Burgess L, Skinner K, Rose R, Colt MJ, Diamond MP. A hyaluronate based gel for the prevention of postsurgical adhesions: Evaluation in two animal species. Fertil Steril, 1996; 66:814-821 which are herein incorporated by reference for material related to post surgical adhesion.)

Recently, GAG molecules have been chemically modified (Luo, Y., Kirker, K. R., and Prestwich, G. D. (2000) Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release* 69, 169-184; Pouyani, T., Harbison, G. S., and Prestwich, G. D. (1994) Novel hydrogels of hyaluronic acid: synthesis, surface morphology, and solid-state NMR. J Am Chem Soc 116, 7515-7522; and Pouyani, T., and Prestwich, G. D. (1994) Functionalized derivatives of hyaluronic acid oligosaccharides - drug carriers and novel biomaterials. Bioconjugate Chemistry 5, 339-347 which are herein incorporated by reference in their entireties at least for material related to chemically modified GAGs and HA.).

V. SUMMARY OF THE INVENTION

In accordance with the purpose(s) of this invention, as embodied and broadly described herein, this invention, in one aspect, relates to hydrogel films and methods of making and using these films.

Additional advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The advantages of the invention will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

VI. BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate different embodiments of the invention and together with the description, serve to explain the principles of the invention.

Fig. 1A shows structures of GAG and chondroitins.

Fig. 1B shows preparation of HA hydrogel film by crosslinking hyaluronic acid converted to the adipic dihydrazide derivative and then crosslinked with poly(ethylene glycol)-propiondialdehyde.

Fig. 2 shows percent wound contraction for the *in vivo* wound healing assays. Results are shown for the two assay groups, HA film plus Tegaderm™ and CS film plus Tegaderm™, and the control group, Tegaderm™ only, (mean ± s.d, n=6).

Fig. 3 shows percent wound re-epithelialization for *in vivo* wound healing

assays. Results are shown for the two assay groups, HA film plus Tegaderm™ and CS film plus Tegaderm™, and the control group, Tegaderm™ only, (mean ± s.d, n=6). (*) Significant at $p<0.001$ vs. Tegaderm™ only and (#) significant at $p<0.05$ vs. Tegaderm™ only.

Fig. 4 shows a diagram of an *in vivo* pig assay.

Fig. 5 shows a diagram of histology methods; (a) Front view of the division of one wound into four equal pieces, with the arrows indicating the edge sections for histological evaluation, (b) Side view of one piece of the wound. Dark represents the wound, while light represents the surrounding tissue.

Fig. 6 shows a diagram of the densitometry method.

Fig. 7 shows percent re-epithelialization of wounds treated with either an HA film+Tegaderm™ or Tegaderm™ alone on days 3 and 7 post surgery. (*) Significant at $p<0.05$ vs. Tegaderm only.

Fig. 8 shows a bar graph of microvessel density.

Fig. 9 shows the total release of bFGF from an ethylene oxide sterilized HA film.

Fig. 10 shows the total release of bFGF from an unsterilized HA film.

Fig. 11 shows HA Oxidation of HA by Periodate (Note: these dialdehydes would be produced in multiple locations along the HA backbone, leading to a polyaldehyde species).

Fig. 12 shows a structural depiction of an HA-ADH chain (horizontal, top) cross-linked by an HA-polyaldehyde chain.

VII. DETAILED DESCRIPTION

The present invention may be understood more readily by reference to the following detailed description of preferred embodiments of the invention and the Examples included therein and to the Figures and their previous and following description.

Before the present compounds, compositions, and/or methods are disclosed and described, it is to be understood that this invention is not limited to specific synthetic methods, specific reagents, or to particular molecular biology or other techniques, as such may, of course, vary, unless it is otherwise indicated. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

A. Definitions

The term "hydrogel film" is intended to mean a macromolecular network which is capable of swelling in aqueous solution.

According to the present invention, an isolated or biologically pure, protein or nucleic acid molecule is a compound that has been removed from its natural milieu. As such, "isolated" and "biologically pure' do not necessarily reflect the extent to which the compound has been purified unless specific levels of purity are indicated. An isolated compound of the present invention can be obtained from its natural source, can be produced using molecular biology techniques or can be produced by chemical synthesis.

As used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an aromatic compound" includes mixtures of aromatic compounds, reference to "a pharmaceutical carrier" includes mixtures of

two or more such carriers, and the like. A polysaccharide means one or more polysaccharides.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another ·diment includes from the one particular value and/or to the other particular

Similarly, when values are expressed as approximations, by use of the ·ntecedent "about," it will be understood that the particular value forms another ·diment. It will be further understood that the endpoints of each of the ranges ·gnificant both in relation to the other endpoint, and independently of the other endpoint.

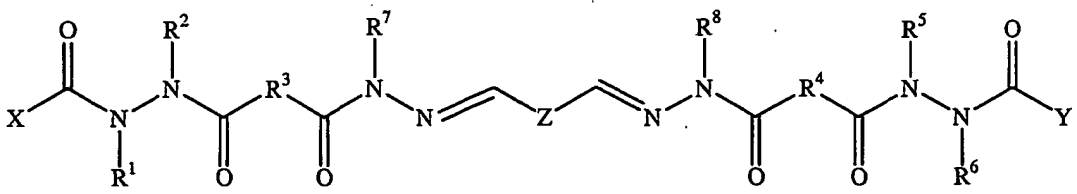
In this specification and in the claims which follow, reference will be made to a number of terms which shall be defined to have the following meanings:

"Optional" or "optionally" means that the subsequently described event or circumstance may or may not occur, and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, the phrase "optionally substituted lower alkyl" means that the lower alkyl group may or may not be substituted and that the description includes both unsubstituted lower alkyl and lower alkyl where there is substitution.

Reference will now be made in detail to the present preferred embodiment(s) of the invention, an example(s) of which is [are] illustrated in the accompanying drawings. Wherever possible, the same reference numbers and letters are used throughout the drawings to refer to the same or like parts.

B. Sampling of disclosed embodiments

In one embodiment, the invention relates to a hydrogel film comprising a polymer, wherein the polymer has at least one unit having the formula I



I

wherein

X and Y are a polysaccharide residue; and

Z, R1, R2, R3, R4, R5, R6, R7, and R8 are, independently, hydrogen, a polysaccharyl group, a substituted or unsubstituted hydrocarbyl group, a substituted or unsubstituted heterohydrocarbyl group, or a polyether group, wherein Z, R3, and R4 are not hydrogen.

The invention also relates to a hydrogel film comprising a compound having the formula II:

[Ax-By-Ax]j II

wherein

A is a glycosaminoglycan having at least one hydrazide group;

B is a dialdehyde crosslinker;

x is the number of glycosaminoglycan molecules, which is a whole number in a range of 1 to 100 molecules;

y is the number of dialdehyde crosslinker molecules, which is a whole number in the range of 1 to 10 molecules; and

j is the number of crosslinked glycosaminoglycan-dialdehyde crosslinker-glycosaminoglycan units, which is a whole number in the range of 10 units to 100 million units.

The invention also relates to a hydrogel film produced by the process comprising reacting (1) a modified polysaccharide having at least one hydrazide group with (2) a polyaldehyde.

The invention yet further provides a method for producing a hydrogel film of the invention, comprising reacting (1) a modified polysaccharide having at least one hydrazide group with (2) a polyaldehyde.

The invention also provides a pharmaceutical composition comprising a pharmaceutically-acceptable compound and the hydrogel film of the invention.

In another embodiment, the invention provides a method for producing a pharmaceutical composition, comprising admixing a pharmaceutically-acceptable compound with the hydrogel film of the invention.

In yet another embodiment, the invention relates to a method for producing a pharmaceutical composition, comprising

(a) admixing a pharmaceutically-acceptable compound with a modified polysaccharide having at least one hydrazide group, and

reacting the modified polysaccharide in the admixture of step (a) with a polyaldehyde.

The invention also relates to a method for improving wound healing in a mammal in need of wound healing, comprising contacting the wound of a mammal

with a hydrogel film of the invention.

The invention further provides a method for delivering at least one pharmaceutically-acceptable compound to a patient in need of such delivery, comprising contacting at least one tissue capable of receiving the pharmaceutically-acceptable compound with a pharmaceutical composition of the invention.

The invention also provides a method for purifying a modified polysaccharide having at least one hydrazide group, comprising performing a dialysis step.

C. Components Used to Prepare Hydrogel Films

Disclosed are the components used to prepare the hydrogel films and the pharmaceutical compositions of the invention. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a number of polysaccharides are disclosed and discussed and a number of modifications that can be made to a number of molecules including polysaccharides are discussed, specifically contemplated is each and every combination and permutation of polysaccharide and the modifications that are possible unless specifically indicated to the contrary. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if it each is not individually recited each is individually and collectively contemplated meaning combinations, A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are considered disclosed. Likewise, any subset or combination of these is also disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E would be considered disclosed. This concept applies to all

aspects of this application including, but not limited to, steps in methods of making and using.

1. Polysaccharides

Polysaccharides useful in the present invention should have at least one carboxylic acid group that can react with a dihydrazide to produce a modified polysaccharide. The synthesis of modified polysaccharides is discussed below. In one embodiment, the polysaccharide is a GAG. A GAG is one molecule with many alternating subunits. For example, HA is (GlcNAc-GlcUA-)x. Other GAGs are sulfated at different sugars. Generically, GAGs are represented by the formula A-B-A-B-A-B, where A is a uronic acid and B is an aminosugar that is either O- or N-sulfated. Any uronic acid containing natural or synthetic polymer is included within the scope of the present invention. The number of disaccharide units may be any that are useful in the present invention.

There are many different types of GAGs, having commonly understood structures, which, for example, are within the disclosed compositions, such as chondroitin sulfate, dermatan, heparan, heparin, dermatan sulfate, and heparan sulfate. Any GAG known in the art can be used in the present invention. Glycosaminoglycans can be purchased from Sigma, and many other biochemical suppliers. Alginic acid, pectin, and carboxymethylcellulose are among other carboxylic acid containing polysaccharides useful in the invention.

Hyaluronic acid is a non-sulfated GAG. Hyaluronic acid is a well known, naturally occurring, water soluble polysaccharide composed of two alternatively linked sugars, D-glucuronic acid and N-acetylglucosamine. The polymer is hydrophilic and highly viscous in aqueous solution at relatively low solute concentrations. It often occurs naturally as the sodium salt, sodium hyaluronate. Methods of preparing commercially available hyaluronic acid and salts thereof are

well known. Hyaluronic acid can be purchased from Clear Solutions Biotechnology, Inc. (Stonybrook, NY), Pharmacia Inc., Sigma Inc., and many other suppliers. For high molecular weight hyaluronic acid it is often in the range of 100-10,000 disaccharide units.

2. Modified Polysaccharide

The polysaccharides containing at least one carboxylic acid group can be modified with hydrazide compounds. The term "modified polysaccharide" refers to a polysaccharide having at least one hydrazide group. The synthesis of the modified polysaccharides will be discussed below. Dihydrazides that can be used to modify the polysaccharide are represented by formula III:

$$H_2N\text{--}NH\text{--}CO\text{--}E\text{--}CO\text{--}NH\text{--}NH_2 \quad \text{(III)}$$

wherein E is hydrocarbyl such as alkyl, aryl, alkylaryl or arylalkyl or E is heterohydrocarbyl, which also includes oxygen, sulfur, and/or nitrogen atoms in addition to carbon atoms. The alkyl group may be branched or unbranched and contain one to 20 carbons or other carbon-sized atoms, preferably 2 to 10, more preferably 4 to 8 carbons or carbon-sized heteroatoms, such as oxygen, sulfur or nitrogen. The alkyl group may be fully saturated or may contain one or more multiple bonds. The carbon atoms of the alkyl may be continuous or separated by one or more functional groups such as an oxygen atom, a keto group, an amino group, an oxycarbonyl group and the like. The alkyl group may be substituted with one or more aryl groups. The alkyl group may in whole or in part, be in form of rings such as cyclopentyl, cyclohexyl, and the like. Any of the alkyl groups described above may have double or triple bond(s).

Any of the hydrocarbyl groups can be used as a heterocarbyl group, wherein the alkyl or aryl group contains a heteroatom such as oxygen, sulfur, or nitrogen incorporated within the chain or ring. Moreover, any of the carbon atoms of the

alkyl group may be separated from each other or from the dihydrazide moiety with one or more groups such as carbonyl, oxycarbonyl, amino, and also oxygen and sulfur atoms singly or in a configuration such as --S--S--, --O--CH_2 --CH_2 --O--, S--S--CH_2 --CH_2 -- and $NH(CH_2)_nNH$--, where n is from 1 to 20.

Aryl substituents are typically substituted or unsubstituted phenyl, but may also be any other aryl group such as pyrrolyl, furanyl, thiophenyl, pyridyl, thiazoyl, etc. An inorganic, alkyl or other aryl group including halo, hydroxy, amino, thioether, oxyether, nitro, carbonyl, etc may further substitute the aryl group.

The alkylaryl or arylalkyl groups may be a combination of alkyl and aryl groups as described above. These groups may be further substituted as described above.

E in formula II, H_2N--NH--CO--NH--E--CO--NH--NH_2, can be hydrocarbyl, heterocarbyl, substituted hydrocarbyl substituted heterocarbyl and the like. The term "hydrocarbyl" as used herein means the monovalent moiety obtained upon removal of a hydrogen atom from a parent hydrocarbon. Representative of hydrocarbyl are alkyl of 1 to 20 carbon atoms, inclusive, such as methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, undecyl, decyl, dodecyl, octadecyl, nonodecyl, eicosyl, heneicosyl, docosyl, tricosyl, tetracosyl, pentacosyl and the isomeric forms thereof; aryl of 6 to 12 carbon atoms, inclusive, such as phenyl, tolyl, xylyl, naphthyl, biphenyl, tetraphenyl and the like; aralkyl of 7 to 12 carbon atoms, inclusive, such as benzyl, phenethyl, phenpropyl, phenbutyl, phenhexyl, napthoctyl and the like; cycloalkyl of 3 to 8 carbon atoms, inclusive, such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl and the like; alkenyl of 2 to 10 carbon atoms, inclusive, such as vinyl, allyl, butenyl, pentenyl, hexenyl, octenyl, nonenyl, decenyl, undececyl, dodecenyl, tridecenyl, pentadecenyl, octadecenyl, pentacosynyl and isomeric forms thereof. Preferably, the hydrocarbyl group has 1 to 20 carbon atoms, inclusive.

The term "substituted hydrocarbyl and heterocarbyl" as used herein means the hydrocarbyl or heterocarbyl moiety as previously defined wherein one or more hydrogen atoms have been replaced with a chemical group, which does not adversely affect the desired preparation of the modified polysaccharide. Representative of such groups are amino, phosphino, quaternary nitrogen (ammonium), quaternary phosphorous (phosphonium), hydroxyl, amide, alkoxy, mercapto, nitro, alkyl, halo, sulfone, sulfoxide, phosphate, phosphite, carboxylate, carbamate groups and the like.

The invention also contemplates that E can be a polysaccharyl group or a polyether group.

Generally, to obtain dihydrazides, two hydroxy groups of a dicarboxylic acid are substituted with NH_2 NH_2 yielding the dihydrazide. Examples of dicarboxylic acids include, but are not limited to, maleic acid, fumaric acid, and aromatic dicarboxylic acids, such as terephthalic acid and isophthalic acid.

In one embodiment, aliphatic dihydrazides, where E is an alkyl group, may have the formula IV:

$$NH_2\ NHCO(CH_2)_{n'}\ CONHNH_2 \quad (IV)$$

wherein n' can be any length but is preferably from 1 to 20. Aliphatic dihydrazides useful in the invention include, but are not limited to, succinic (butandioic) (n'=2), adipic (hexanedioic) (n'=4), suberic (octanedioic) (n'=6), oxalic (ethanedioic) (n'=0), malonic (propanedioic) (n'=1), glutaric (pentanedioic) (n'=3), pimelic (heptanedioic) (n'=5), azelaic (nonanedioic) (n'=7), sebacic (decanedioic) (n'=8), dodecanedioic, (n'=10), brassylic (tridecanedioic), (n'=11), (etc. up to n'=20).

In a preferred embodiment, adipic dihydrazide, suberic dihydrazide, and butandioic dihydrazide are used to prepare the modified polysaccharide. Adipic dihydrazide can be purchased from Aldrich Chemical Co. (Milwaukee, WI). Also

preferred are phthalic dihydrazide and dihydrazides with E containing oxa, thio, amino, disulfide (--CH_2 --S--S--CH_2 --), --$S(CH_2)_2$ S--, --$O(CH_2)_n$ O-- or -- $NH(CH_2)_nNH$-- (n=2 to 4) groups.

It is preferred that the dihydrazides are at least partially soluble in water. The dihydrazides are also weak bases or weak acids having a pK_a for the protonated form, less than about 8, preferably in the range of 1 to 7 and most preferably 2 to 6.

It will be understood that the term pK_a is used to express the extent of dissociation or the strength of weak acids, so that, for example, the pK_a of the protonated amino group of amino acids is in the range of about 12-13 in contrast to the pK_a of the protonated amino groups of the dihydrazides useful herein which is less than about 7.

The conversion of polysaccharide to the corresponding modified polysaccharides (*i.e.*, their dihydrazide derivatives) generally involves reacting the polysaccharide with a dihydrazide. In particular, the dihydrazide reacts with a carboxylic acid group present on the polysaccharide. The reaction is preferably carried out under mild conditions at a pH of about 2 to 8, preferably about 3 to 6. In one embodiment, the polysaccharide is dissolved in water, which may also contain water-miscible solvents including, but not limited to, dimethylformamide, dimethylsulfoxide, and hydrocarbyl alcohols, diols, or glycerols.

The number of dihydrazide groups present on the modified polysaccharide used will vary depending upon the amounts of dihydrazide and polysaccharide used. In one embodiment, 1% to 99%, 10% to 90%, 20% to 80%, 30% to 70%, or 40% to 50% of the carboxylic acid groups present on the polysaccharide are converted to the dihydrazide. In one embodiment, at least one molar equivalent of dihydrazide per molar equivalent of polysaccharide is added. In other embodiments, for maximum percentage functionalization, a large molar excess of the dihydrazide (*e.g.*, 10-100 fold) dissolved in water or aqueous-organic mixture is added and the pH of the

reaction mixture is adjusted by the addition of dilute acid, *e.g.*, HCl. A sufficient molar excess (*e.g.*, 2 to 100 fold) of carbodiimide reagent dissolved in water, in any aqueous-organic mixture, or finely-divided in solid form is then added to the reaction mixture.

An increase in pH may be observed after the addition of the carbodiimide and additional dilute HCl or other dilute acids may be added to adjust the pH. The reaction is allowed to proceed at a temperature of about 0°C to about 100° C (*e.g.*, just above freezing, 0° C, to just below boiling (100° C), preferably at or near ambient temperatures for purposes of convenience. The time of the reaction is from about 0.5 to about 48 hours, preferably about one to about five hours with periodic testing and adjusting of the pH until no further change in pH is observed.

Any of the polysaccharides and dihydrazide compounds discussed above can be reacted with one another to produce the modified polysaccharide. In one embodiment, the modified polysaccharide comprises the reaction product between a GAG and a dihydrazide compound. In one preferred embodiment, the modified polysaccharide comprises the reaction product between adipic dihydrazide and hyaluronic acid (HA-ADH). In another preferred embodiment, the modified polysaccharide comprises the reaction product between adipic dihydrazide and chondroitin sulfate.

Prior to reacting the modified polysaccharide with the polyaldehyde crosslinker, the modified polysaccharide can be further purified to enhance the crosslinking efficiency of the reaction with the polyaldehyde. The invention provides a method for purifying the modified polysaccharide via dialysis. A series of dialysis steps can be performed in order to increase the overall purity of the modified polysaccharide.

The dialysis steps will vary depending upon the nature of the modified polysaccharide. In particular, the solvent that is used to perform the dialysis step can

increase the purity of the modified polysaccharide. For example, the solvent can be water, aqueous alcohol, or an aqueous salt. Any salt or alcohol known in the art is useful in this method, and will vary depending upon the modified polysaccharide.

In one embodiment, a method for purifying HA-ADH comprises dialyzing HA-ADH against an aqueous salt, then successively against alternating solutions of aqueous alcohol and water. In this embodiment, the aqueous salt is NaCl and the alcohol is ethanol. The invention also contemplates the optional steps of centrifuging the solution and lyophilizing the supernatant after the dialysis steps.

In a more particular embodiment, the method for preparing and purifying HA-ADH comprises the consecutive steps of: (1) dissolving hyaluronic acid in water; (2) adding adipic dihydrazide to hyaluronic acid while stirring; (3) adjusting the pH to a range of 4.5 to 5.0; (4) adding 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide in solid form; (5) maintaining a pH range of 4.5 to 5.0; (6) stopping the reaction by raising the pH to 7.0; (7) transferring the reaction mixture to dialysis tubing with a molecular weight cut-off of 3,500 that has been soaked in water at room temperature for 3-4 hours and then rinsed; (8) dialyzing against 100 mM NaCl for 60 hours; (9) dialyzing against 1:3 EtOH-H_2O (volume/volume); (10) dialyzing against pure water; and (11) dialyzing against 1:3 EtOH-H_2O (volume/volume).

3. Polyaldehyde Crosslinker

In the present invention, a polyaldehyde crosslinker is reacted with the modified GAG to produce the hydrogel film of the invention. A polyaldehyde is a compound that has two or more aldehyde groups [C(O)H]. In certain embodiments the aldehyde is a dialdehyde composition.

Any compound possessing two or more aldehyde groups can be used in the present invention as the polyaldehyde crosslinker. In one embodiment, the

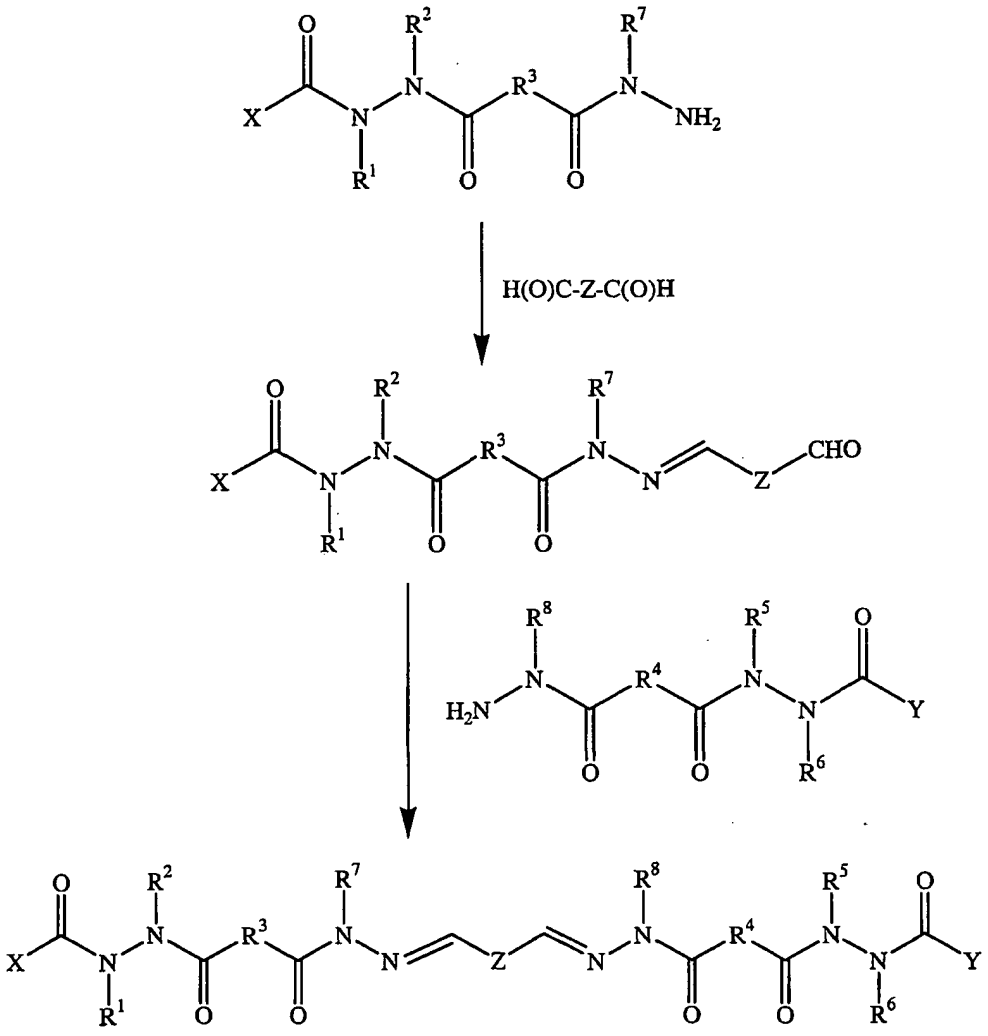
polyaldehyde can be substituted or unsubstituted hydrocarbyl or substituted or unsubstituted heterohydrocarbyl. In another embodiment, the polyadlehyde can contain a polysaccharyl group or a polyether group. In a further embodiment, the polyaldehyde can be a dendrimer or peptide. In the present invention, a polyether dialdehyde such as poly(ethylene glycol) propiondialdehyde (PEG) is useful in the present invention. PEG can be purchased from many commercial sources, such as Shearwater Polymers, Inc. (Huntsville, AL). In another embodiment, the polyaldehyde is glutaraldehyde.

Alternatively, polyaldehydes of the invention can be prepared by the oxidation of terminal polyols or polyepoxides possessing two or more hydroxy or epoxy groups, respectively, using techniques known in the art.

D. Methods of Making Hydrogel Films

Also provided are methods for making the hydrogel films of the invention. The method generally involves reacting the modified polysaccharide with the polyaldehyde crosslinker in the presence of a solvent. In particular, the aldehyde group of the polyaldehyde reacts with the hydrazide group of a modified polysaccharide to produce a new carbon-nitrogen double bond, which is depicted in Scheme 1. The second aldehyde group is then capable of reacting with the hydrazide group of a second modified polysaccharide to produce another carbon-nitrogen double bond to produce the unit depicted in Formula I.

SCHEME 1



I

In view of Scheme 1, it is possible to crosslink two or more modified polysaccharides to produce a matrix. "Crosslinking" is defined as the ability of the

polyaldehyde to react with two or more independent modified polysaccharides to produce a pore-containing matrix, wherein pharmaceutically-acceptable compounds can be physically or chemically incorporated. Although the polyaldehyde is intended to react with hydrazide groups on different modified polysaccharides, it is also possible that the polyaldehyde can react with two or more hydrazide groups present on the same modified polysaccharide.

It also evident in Scheme 1 that the modified polysaccharides can be different or the same. Thus, in one embodiment, X and Y can be the same polysaccharide residue. In another embodiment, X and Y can be different polysaccharide residues. The term "residue" is a section of pre-existing molecule that forms a portion of a new molecule. For example, the polysaccharide can be depicted as X-COOH, where X is the remainder of the polysaccharide molecule. When X-COOH reacts with a dihydrazide to produce the modified polysaccharide, X remains the same and is part of the modified polysaccharide. In other words, X is the residue of the original GAG (X-COOH). In one embodiment, X and Y are, independently, a residue of chondroitin sulfate, dermatan, heparan, heparin, dermatan sulfate, heparan sulfate, alginic acid, pectin, or carboxymethylcellulose. In another embodiment, X and Y are a residue of hyaluronic acid.

The overall number of crosslinks and the number of different modified polysaccharides that are cross linked together are dependent on the number of reactive aldehyde groups in the polyaldehyde and dihydrazide groups present on the modified polysaccharide. At a minimum, there is at least one crosslink (*i.e.*, unit) having the formula I in the hydrogel films of the invention. In one embodiment, 1% to 100%, 10% to 90%, 30% to 80%, or 40% to 70% of the dihydrazide groups are crosslinked with the polyaldehyde. In one embodiment, hydrogel film has from 10 to 10,000,000 units having the formula I. In a preferred embodiment the hydrogel has from 10 to 10,000 units having the formula I.

In one embodiment, adipic dihydrazide (ADH) will crosslink when it modifies the uronic acid in 1%-99% of the glycosaminoglycan or 1-50%. In general the modification of the carboxylic acid containing polysaccharide such as GAG (for example HA) can contain 10-90% or 20-80% or 30-70% or 40-60% or about 50% derivatization and the derivatized polysaccharide can contain greater than 10% or 20% or 30% or 40% or 50% or 60% or 70% or 80% or 90% or 99% crosslinking. For example, a hyaluronic acid (HA) with 5000 disaccharide units (normal high MW HA) has 5000 carboxylic acid groups available. A 1% modification means that there are 50 ADHs per HA molecule, 10% would be 500 ADH/HA, etc. Thus, even at low modification levels, there are some 50 sites per modified GAG molecule to form crosslinks.

Referring to Formula I, in one embodiment, Z is a polyether. In another embodiment, R^1, R^2, R^5, R^6, R^7, and R^8 are hydrogen. In another embodiment, R^3 and R^4 are $(CH_2)_n$, wherein n is from 1 to 20, preferably 2 to 4. In a more preferred embodiment, X and Y are a residue of hyaluronic acid, Z is a polyethylene ether, R^1, R^2, R^5, and R^6 are hydrogen, and R^3 and R^4 is $(CH_2)_4$. In another embodiment, the hydrogel film is produced by reacting (1) a modified polysaccharide comprising the reaction product between adipic dihydrazide and hyaluronic acid and (2) a poly(ethylene glycol) propiondialdehyde.

After the reaction between the polyaldehyde and the modified polysaccharide is complete, the solvent present in the hydrogel film may be evaporated by any method known in the art such as air-drying, rotary evaporation at low pressure and/or lyophilization. Preferably at least 80% of the solvent contained within the hydrogel film will evaporate. More preferred is a state where at least 90% of the solvent has evaporated from the hydrogel film. However, most preferred are hydrogel films in which at least 98% of the solvent has evaporated.

In one embodiment, the reaction solvent is water. In addition, small amounts

of water miscible organic solvents, such as an alcohol or DMF or DMSO, may be used as well. It is preferred that the cross-linking reaction be performed at room temperature, for example, 25 °C, but the cross-linking reaction can be performed within a range of temperatures from below 4 °C to above 90 °C but typically would be performed at between 4 °C and 60 °C, more typically between 4 °C and 50 °C, and more preferably at 4 °C or 30 or 37 degrees. The reaction will also work at a variety of pHs between, for example, pH from 3 to10, or pH from 4 to 9, or pH from 5 to 8, or preferably at neutral pH.

E. Pharmaceutical Compositions

In one embodiment the hydrogel film may comprise at least one pharmaceutically-acceptable compound. The resulting pharmaceutical composition can provide a system for sustained, continuous delivery of drugs and other biologically-active agents to tissues adjacent to or distant from the application site. The biologically-active agent is capable of providing a local or systemic biological, physiological or therapeutic effect in the biological system to which it is applied. For example, the agent may act to control infection or inflammation, enhance cell growth and tissue regeneration, control tumor growth, and enhance bone growth, among other functions.

Additionally, hydrogel films of the invention may contain combinations of two or more pharmaceutically-acceptable compounds. Hydrogel films which contain five or fewer pharmaceutically-acceptable compounds are preferred. More preferred are hydrogel films which contain two or fewer pharmaceutically-acceptable compounds. Hydrogel films which contain one pharmaceutically-acceptable compound are most preferred.

The pharmaceutically-acceptable compounds can include substances capable of preventing an infection systemically in the biological system or locally at the

defect site, as for example, anti-inflammatory agents such as pilocarpine, hydrocortisone, prednisolone, cortisone, diclofenac sodium, indomethacin, 6∝-methyl-prednisolone, corticosterone, dexamethasone, prednisone, and the like; antibacterial agents such as penicillin, cephalosporins, bacitracin, tetracycline, doxycycline, gentamycin, chloroquine, vidarabine, and the like; analgesic agents such as salicylic acid, acetaminophen, ibuprofen, naproxen, piroxicam, flurbiprofen, morphine, and the like; local anesthetics such as cocaine, lidocaine, benzocaine, and the like; immunogens (vaccines) for stimulating antibodies against hepatitis, influenza, measles, rubella, tetanus, polio, rabies, and the like; peptides such as leuprolide acetate (an LH-RH agonist), nafarelin, and the like. All compounds are available from Sigma Chemical Co. (Milwaukee, WI).

Additionally, a substance or metabolic precursor which is capable of promoting growth and survival of cells and tissues or augmenting the functioning of cells is useful, as for example, a nerve growth promoting substance such as a ganglioside, a nerve growth factor, and the like; a hard or soft tissue growth promoting agent such as fibronectin (FN), human growth hormone (HGH), a colony stimulating factor, bone morphogenic protein, platelet-derived growth factor (PDGF), insulin-derived growth factor (IGF-I, IGF-II), transforming growth factor-alpha (TGF-alpha), transforming growth factor-beta (TGF-beta), epidermal growth factor (EGF), fibroblast growth factor (FGF), interleukin-1 (IL-1), vascular endothelial growth factor (VEGF) and keratinocyte growth factor (KGF), dried bone material, and the like; and antineoplastic agents such as methotrexate, 5-fluorouracil, adriamycin, vinblastine, cisplatin, tumor-specific antibodies conjugated to toxins, tumor necrosis factor, and the like.

Other useful substances include hormones such as progesterone, testosterone, and follicle stimulating hormone (FSH) (birth control, fertility-enhancement), insulin, and the like; antihistamines such as diphenhydramine, and the like; cardiovascular agents such as papaverine, streptokinase and the like; anti-ulcer

agents such as isopropamide iodide, and the like; bronchodilators such as metaproternal sulfate, aminophylline, and the like; vasodilators such as theophylline, niacin, minoxidil, and the like; central nervous system agents such as tranquilizer, B-adrenergic blocking agent, dopamine, and the like; antipsychotic agents such as risperidone, narcotic antagonists such as naltrexone, naloxone, buprenorphine; and other like substances. All compounds are available from Sigma Chemical Co. (Milwaukee, WI).

In a preferred embodiment, the pharmaceutically-acceptable compound is a steroid. In another embodiment, the pharmaceutical composition comprises a steroid and a hydrogel film comprised of the reaction product between hyaluronic acid/adipic dihydrazide (HA-ADH) and poly(ethylene glycol) propiondialdehyde.

The pharmaceutical compositions can be prepared using techniques known in the art. In one embodiment, the composition is prepared by admixing a hydrogel film of the invention with a pharmaceutically-acceptable compound. The term "admixing" is defined as mixing the two components together so that there is no chemical reaction or physical interaction. The term "admixing" also includes the chemical reaction or physical interaction between the hydrogel film and pharmaceutically-acceptable compound. Covalent bonding to reactive therapeutic drugs, e.g., those having reactive carboxyl groups, can be undertaken on the hydrogel film. For example, first, carboxylate-containing chemicals such as anti-inflammatory drugs ibuprofen or hydrocortisone-hemisuccinate can be converted to the corresponding N-hydroxysuccinimide (NHS) active esters and can further react with the NH2 group of the dihydrazide-modified polysaccharide. Second, non-covalent entrapment of a pharmacologically active agent in cross-linked polysaccharide is also possible. Third, electrostatic or hydrophobic interactions can facilitate retention of a pharmaceutically-acceptable compound in the modified polysaccharide. For example, the hydrazido group can non-covalently interact, e.g., with carboxylic acid-containing steroids and their analogs, and anti-inflammatory

drugs such as Ibuprofen (2-(4-iso-butylphenyl) propionic acid). The protonated hydrazido group can form salts with a wide variety of anionic materials such as proteins, heparin or dermatan sulfates, oligonucleotides, phosphate esters, and the like.

In another embodiment, the pharmaceutically-acceptable compound is admixed with the modified polysaccharide, followed by reacting the modified polysaccharide with a polyaldehyde. This embodiment also covers the possibility of the pharmaceutically-acceptable compound chemically reacting or physically interacting with the modified polysaccharide.

It will be appreciated that the actual preferred amounts of active compound in a specified case will vary according to the specific compound being utilized, the particular compositions formulated, the mode of application, and the particular situs and mammal being treated. Dosages for a given host can be determined using conventional considerations, e.g. by customary comparison of the differential activities of the subject compounds and of a known agent, e.g., by means of an appropriate conventional pharmacological protocol. Physicians and formulators, skilled in the art of determining doses of pharmaceutical compounds, will have no problems determining dose according to standard recommendations (Physicians Desk Reference, Barnhart Publishing (1999).

Therapeutic compositions of the present invention can be formulated in any excipient the biological system or entity can tolerate. Examples of such excipients include water, saline, Ringer's solution, dextrose solution, Hank's solution, and other aqueous physiologically balanced salt solutions. Nonaqueous vehicles, such as fixed oils, vegetable oils such as olive oil and sesame oil, triglycerides, propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate may also be used. Other useful formulations include suspensions containing viscosity enhancing agents, such as sodium carboxymethylcellulose, sorbitol, or dextran.

Excipients can also contain minor amounts of additives, such as substances that enhance isotonicity and chemical stability. Examples of buffers include phosphate buffer, bicarbonate buffer and Tris buffer, while examples of preservatives include thimerosol, cresols, formalin and benzyl alcohol.

Pharmaceutical carriers are known to those skilled in the art. These most typically would be standard carriers for administration to humans, including solutions such as sterile water, saline, and buffered solutions at physiological pH.

Molecules intended for pharmaceutical delivery may be formulated in a pharmaceutical composition. Pharmaceutical compositions may include carriers, thickeners, diluents, buffers, preservatives, surface active agents and the like in addition to the molecule of choice. Pharmaceutical compositions may also include one or more active ingredients such as antimicrobial agents, antiinflammatory agents, anesthetics, and the like.

The pharmaceutical composition may be administered in a number of ways depending on whether local or systemic treatment is desired, and on the area to be treated. Administration may be topically (including ophthalmically, vaginally, rectally, intranasally).

Preparations for administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles, if needed for collateral use of the disclosed compositions and methods, include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles, if needed for collateral use of the disclosed compositions and methods, include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and

the like.

Formulations for topical administration may include ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable.

Dosing is dependent on severity and responsiveness of the condition to be treated, but will normally be one or more doses per day, with course of treatment lasting from several days to several months or until one of ordinary skill in the art determines the delivery should cease. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates.

F. Methods of Using

The disclosed hydrogel films can be used for a variety of uses related to drug delivery, small molecule delivery, wound healing, burnhealing, and tissue regeneration. The disclosed compositions are useful for situations which benefit from a hydrated, pericellular environment in which assembly of other matrix components, presentation of growth and differentiation factors, cell migration, or tissue regeneration are desirable.

1. Methods for delivery of substances

The present hydrogel films and disclosed compositions can be placed directly in or on any biological system without purification as it is composed of only two biocompatible polymers. Examples of sites the hydrogel film may be placed include soft tissue such as muscle or fat; hard tissue such as bone; areas of tissue regeneration; a void space such as periodontal pocket; surgical incision or other formed pocket or cavity; a natural cavity such as the oral, vaginal, rectal or nasal

cavities, the cul-de-sac of the eye, and the like; and other sites into or onto which the hydrogel film may be placed including a skin surface defect such as a cut, scrape or burn area. The present hydrogel films are biodegradeable and naturally occurring enzymes will act to degrade them over time. Components of the hydrogel film may be "bioabsorbable" in that the components of the hydrogel film will be broken down and absorbed within the biological system, for example, by a cell, tissue and the like. Additionally, the hydrogel films, especially hydrogel films that have not been rehydrated, may be applied to a biological system to absorb fluid from an area of interest.

The hydrogel films of this invention may be used as a carrier for a wide variety of releasable biologically active substances having curative or therapeutic value for human or non-human animals. Many of these substances which can be carried by the hydrogel film are discussed above. Included among biologically active materials which are suitable for incorporation into the gels of the invention are therapeutic drugs, e.g., anti-inflammatory agents, anti-pyretic agents, steroidal and non-steroidal drugs for anti-inflammatory use, hormones, growth factors, contraceptive agents, antivirals, antibacterials, antifungals, analgesics, hypnotics, sedatives, tranquilizers, anti-convulsants, muscle relaxants, local anesthetics, antispasmodics, antiulcer drugs, peptidic agonists, sympathiomimetic agents, cardiovascular agents, antitumor agents, oligonucleotides and their analogues and so forth. A biologically active substance is added in pharmaceutically active amounts.

Preferred methods involve using the compositions and methods in conjunction with and for the delivery of growth factors and molecules related to growth factors. For example the growth factors can be a nerve growth promoting substance such as a ganglioside, a nerve growth factor, and the like; a hard or soft tissue growth promoting agent such as fibronectin (FN), human growth hormone (HGH), a colony stimulating factor, bone morphogenic protein, platelet-derived growth factor (PDGF), insulin-derived growth factor (IGF-I, IGF-II), transforming

growth factor-alpha (TGF-alpha), transforming growth factor-beta (TGF-beta), epidermal growth factor (EGF), fibroblast growth factor (FGF), interleukin-1 (IL-1). Preferred growth factors are bFGF and TGF-β. Also preferred are vascular endothelial growth factor (VEGF) and keratinocyte growth factor (KGF).

Preferred are all anti-inflammatories bearing carboxyl groups such as ibuprofen, naproxen, ketoprofen and indomethacin. Other preferred biologically active substances are peptides, which are naturally occurring, non-naturally occurring or synthetic polypeptides or their isosteres, such as small peptide hormones or hormone analogues and protease inhibitors. Also preferred are spermicides, antibacterials, antivirals, antifungals and antiproliferatives such as fluorodeoxyuracil and adriamycin. These substances are all known in the art. Compounds are available from Sigma Chemical Company (St. Louis, MO).

The term therapeutic drugs is intended to include those defined in the Federal Food, Drug and Cosmetic Act. The United States Pharmacopeia (USP) and the National Formulary (NF) are the recognized standards for potency and purity for most common drug products.

In certain methods the pharmaceutically acceptable compound is selected from the group consisting of pilocarpine, hydrocortisone, prednisolone, cortisone, diclofenac sodium, indomethacin, 6∝-methyl-prednisolone, corticosterone, dexamethasone and prednisone. However, methods are also provided wherein delivery of a pharmaceutically-acceptable compound is for a medical purpose selected from the group consisting of delivery of contraceptive agents, treating postsurgical adhesions, promoting skin growth, preventing scarring, dressing wounds, conducting viscosurgery, conducting viscosupplementation, engineering tissue.

The rate of drug delivery depends on the hydrophobicity of the molecule being released. Hydrophobic molecules, such as dexamethazone and prednisone are

released slowly from the hydrogel film as it swells in an aqueous environment, while hydrophilic molecules, such as pilocarpine, hydrocortisone, prednisolone, cortisone, diclofenac sodium, indomethacin, 6∝-methyl-prednisolone and corticosterone, are released quickly. The ability of the hydrogel film to maintain a slow, sustained release of steroidal anti-inflammatories makes this invention extremely useful for wound healing after trauma or surgical intervention. Additionally, the hydrogel film can be used as a barrier system for enhancing cell growth and tissue regeneration.

In certain methods the delivery of molecules or reagents related to angiogenesis and vascularization are achieved. Disclosed are methods for delivering agents, such as VEGF, that stimulate microvascularization. In one embodiment, HA and the combination of HA with VEGF can enhance the process of neovasculogenesis in *in vivo* applications. Also disclosed are methods for the delivery of agents that can inhibit angiogenesis and vascularization, such as those compounds and reagents useful for this purpose disclosed in but not limited to United States Patent Nos 6,174,861 for "Methods of inhibiting angiogenesis via increasing in vivo concentrations of endostatin protein;" 6,086,865 for "Methods of treating angiogenesis-induced diseases and pharmaceutical compositions thereof;" 6,024,688 for "Angiostatin fragments and method of use;" 6,017,954 for "Method of treating tumors using O-substituted fumagillol derivatives;" 5,945,403 for "Angiostatin fragments and method of use;" 5,892,069 "Estrogenic compounds as anti-mitotic agents;" for 5,885,795 for "Methods of expressing angiostatic protein;" 5,861,372 for "Aggregate angiostatin and method of use;" 5,854,221 for "Endothelial cell proliferation inhibitor and method of use;" 5,854,205 for "Therapeutic antiangiogenic compositions and methods;" 5,837,682 for "Angiostatin fragments and method of use;" 5,792,845 for "Nucleotides encoding angiostatin protein and method of use;" 5,733,876 for "Method of inhibiting angiogenesis;" 5,698,586 for "Angiogenesis inhibitory agent;" 5,661,143 for "Estrogenic compounds as anti-mitotic agents;" 5,639,725 for "Angiostatin protein;" 5,504,074

for "Estrogenic compounds as anti-angiogenic agents;" 5,290,807 for "Method for regressing angiogenesis using o-substituted fumagillol derivatives;" and 5,135,919 for "Method and a pharmaceutical composition for the inhibition of angiogenesis" which are herein incorporated by reference for the material related to molecules for angiogenesis inhibition.

2. Methods related to wound healing

The present invention also provides methods to improve wound healing in a mammal in need of such improvement, comprising contacting a hydrogel film of the present invention with a wound of a mammal in need of wound healing improvement. Also provided are methods to deliver at least one pharmaceutically-acceptable compound to a patient in need of such delivery, comprising contacting at least one hydrogel film of the present invention with at least one tissue capable of receiving said pharmaceutically-acceptable compound.

The disclosed compositions can be used for treating a wide variety of tissue defects in an animal, for example, a tissue with a void such as a periodontal pocket, a wound on the skin, a surgical incision, a bone defect, and the like. For example, the hydrogel film can be applied to a defect in bone tissue such as a fracture in an arm or leg bone, a defect in a tooth, and the like. The hydrogel film can also function as a barrier system for guided tissue regeneration by providing a surface over which cells can grow. To enhance regeneration of a hard tissue such as bone tissue, it is preferred that the hydrogel film provides support for new cell growth that will replace the matrix as it becomes gradually absorbed or eroded by body fluids.

The hydrogel film may be delivered onto cells, tissues, and/or organs, for example, by injection, spraying, squirting, brushing, painting, coating, and the like. Delivery can also be via a cannula, catheter, syringe with or without a needle, pressure applicator, pump, and the like. The hydrogel film may be applied onto a

tissue in the form of a film, for example, to provide a film dressing on the surface of the tissue, and/or to adhere to a tissue to another tissue or hydrogel film, among other applications.

The hydrogel film may be used to treat periodontal disease, gingival tissue overlying the root of the tooth can be excised to form an envelope or pocket, and the composition delivered into the pocket and against the exposed root. The hydrogel film may also be delivered to a tooth defect by making an incision through the gingival tissue to expose the root, and then applying the material through the incision onto the root surface by placing, brushing, squirting, or other means.

When used to treat a defect on skin or other tissue, the hydrogel film can be placed on top of the desired area. The hydrogel film is malleable and can be manipulated to conform to the contours of the tissue defect.

The hydrogel film can be applied to an implantable device such as a suture, claps, prosthesis, catheter, metal screw, bone plate, pin, a bandage such as gauze, and the like, to enhance the compatibility and/or performance or function of an implantable device with a body tissue in an implant site. The hydrogel film can be used to coat the implantable device. For example, the hydrogel film could be used to coat the rough surface of an implantable device to enhance the compatibility of the device by providing a biocompatable smooth surface which reduces the occurrence of abrasions from the contact of rough edges with the adjacent tissue. The hydrogel film can also be used to enhance the performance or function of an implantable device. For example, the hydrogel film can be applied to a gauze bandage to enhance its compatibility or adhesion with the tissue to which it is applied. The hydrogel film can also be applied around a device such as a catheter or colostomy that is inserted through an incision into the body to help secure the catheter/colosotomy in place and/or to fill the void between the device and tissue and form a tight seal to reduce bacterial infection and loss of body fluid.

It is understood that the disclosed compositions and methods can be applied to mammals in need of tissue regeneration. For example, cells may be incorporated into the hydrogel for implantation. Preferred mammals to which the compositions and methods apply are mouse, porcine, bovine, ovine, and primates, including apes, chimpanzees, orangatangs, and humans.

When being used in areas related to tissue regeneration such as wound or burn healing, it is not necessary that the disclosed methods and compositions eliminate the need for one or more related accepted therapies. It is understood that any decrease in the length of time for recovery or increase in the quality of the recovery obtained by the recipient of the disclosed compositions or methods has obtained some benefit. It is also understood that the disclosed compositions and methods may be used to prevent or reduce fibrotic adhesions occurring as a result of wound closure as a result of trauma, such surgery. It is also understood that collateral affects provided by the disclosed compositions and hydrogels are desirable but not required, such as improved bacterial resistance or reduced pain etc.

It is understood that a any given particular embodiment of the disclosed compositions and methods can be easily compared to the specific examples and embodiments disclosed herein, including the non- polysaccharide based reagents discussed in the Examples. By performing such a comparison, the relative efficacy of each particular embodiment can be easily determined. Particularly preferred assays for the various uses are those assays which are disclosed in the Examples herein, and it is understood that these assays, while not necessarily limiting, can be performed with any of the compositions and methods disclosed herein.

G. Examples

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds,

compositions and/or methods claimed herein are made and evaluated, and are intended to be purely exemplary of the invention and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.), but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

1. Example 1. Hyaluronic Acid – Adipic Dihydrazide Hydrogel film Preparation

a) Materials

Fermentation-derived hyaluronan (HA, sodium salt, $M_r = 1.5 \times 10^6$) was obtained from Clear Solutions Biotechnology, Inc. (Stony Brook, New York) and used as received. Adipic Dihydrazide and 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide(EDCI) were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as received. Poly(ethylene glycol)-propiondialdehyde (PEG-diald) was obtained from Shearwater Polymers, Inc. (Huntsville, AL) and also used as received.

b) Preparation of HA-ADH

HA (6 g, 15 mmol) was dissolved in 1.2 L of water to give a 5 mg/ml solution. Adipic dihydrazide (110 g, 0.63 mol) was then added to the solution while stirring. Next, EDCI (10g, 52 mmol) was added in solid form. The pH of the reaction was maintained at 4.75 by the addition of 0.1N HCl. The reaction was stopped by addition of 0.1 N NaOH, raising the pH of the reaction mixture to 7.0. Dialysis tubing (MW cutoff 3,500) was soaked in water at room temperature for 3-4 hours and then rinsed. The reaction mixture was transferred to the prewashed

dialysis tubing and dialyzed exhaustively (60 hour against 100 NaCL) followed by dialysis against alternating solutions of 1:3 EtOH-H_2O (v/v) and pure H_2O. The solution was then centrifuged, and the supernatant lyophilized. The purity of the resulting HA-ADH was measured by GPC, and the degree of substitution by ADH was determined by H-NMR.

2. Example 2. Purification of the HA-ADH

Crude HA-ADH was dialyzed against 100 mM NaCl for 60 hours, followed by four cycles of 25% ethanol, followed by water gave GPC-homogenous HA-ADH. Purified HA-ADH was dissolved in deionized water and the degree of substitution determined by integration of the ADH methylene signals using the N-acetyl methyl resonances (δ = 1.95-2.00 ppm) as an internal standard. The degree of substitution of ADH substitution of the HA-ADH used in this Example was 55%, based on the number of available glucoronates modified.

a) Preparation of HA-PEG Hydrogel film

HA-ADH was dissolved in H_2O at a concentration of 5 mg/ml (Solution A). PEG-dialdehyde was dissolved in H_2O at a concentration of 50 mg/ml (Solution B). Solutions A and B were added to a small Petri dish in appropriate ratios to give equimolar equivalents of aldehyde and hydrazide functionalities and the solutions were mixed with gentle swirling. A hydrogel film began to form within 60 seconds. Hydrogel films were successfully produced when the crosslinking agent (PEG-diald) was used in a molar ration of 0.25, 0.5, and 1 relative to ADH. The mixture was agitated on an orbital platform for an additional 24 hours to obtain a solid, uniform hydrogel film. Hydrogel films were stored in an open dish overnight at 37° C to allow solvent evaporation. Evaporation of solvent from the hydrogel film provided flexible, hydratable HA hydrogel film.

3. Example 3. Differential Scanning Calorimeter Analysis of Hydrogel films

a) a) Instruments

Differential Scanning Calorimeter (DSC) analysis was carried out on a Model 911 Differential Scanning Calorimeter.

b) Procedure

The thermal analysis profiles of the dried hydrogel film samples (with or without loading) were obtained as the temperature was increased from room temperature to 55° C at a rate of 10° C/minute under nitrogen.

c) Results

The DSC profiles show that after HA is converted into its ADH derivative, the endothermic and exothermic peak shifted, indicating an altered polymer microstructure. When comparing the HA-ADH and PEG-diald, the crosslinking of the two polymers clearly produced a new material having a microstructure different from its components.

4. Example 4. Scanning Electron Microscopy

a) a) Procedure

HA hydrogel films were gently rinsed with H_2O and air-dried in an incubator at 37° C for 24 hours to give dried hydrogel samples. Swollen film samples were obtained by immersion of the HA hydrogel film disks in distilled H_2O for 15 minutes, freezing quickly on dry ice, followed by lyophilization. HA hydrogel films

subjected to enzymatic degradation were prepared by immersion of the films in pH 7.4 PBS buffer containing hyaluronidase (HAse) (100 U/mL) for 3 days. The films were removed from the HAse solution, rinsed gently with water and dried on PTFE surface at 37C. For comparison, control HA hydrogel films were immersed in pH 7.4 PBS buffer without HAse at 37° C for 3 days and processed as for the enzyme-treated gels. Samples were gold-coated for conductance, and the surfaces of hydrogel films were examined with an SEM. Magnifications were at 6,000x and below.

b) Results

Flat and featureless images indicate that the HA hydrogel films have a condensed structure when dry, whereas highly-porous structures are evident in water-swollen gels. In the hydrogel bioerosion assay, the control sample of HA hydrogel immersed in enzyme-free PBS buffer for 3 days retained an intact, condensed surface structure. In contrast, addition of 100 U/ml of HAse to the buffer produced significant surface erosion of the HA hydrogel. The surface differences of the hydrogel in buffer with or without enzyme clearly demonstrated that HAse can recognize and process the crosslinked HA and that the HA hydrogel films would be expected to be biodegradable *in vivo*.

5. Example 5. Hyaluronic Acid – Adipic Dihydrazide Hydrogel Preparation with Dye-Loading

a) Materials

Amaranth and acridine orange were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used as received.

b) Preparation of HA-PEG hydrogel films with amaranth loading

Amaranth was loaded into the hydrogel films by an *in situ* polymerization method. Amaranth was dissolved in H_2O (10 mg/ml) and then mixed Solution A. The PEG-dialdehyde (50 mg/ml) Solution B was then added to create a hydrogel film containing the amaranth dye. The resulting hydrogel film was obtained by evaporation of solvent with amaranth-loading of 5.0% weight percentage relative to the dry macromolecular components.

c) Preparation of HA-PEG Hydrogel film with acridine orange loading

A solution absorption method was used to load acridine orange onto the HA hydrogel film. A HA hydrogel film was prepared and then immersed in a 1 mg/mL acridine orange/H_2O solution for 60 seconds. The film was then washed with water, placed on a poly(tetrafluoroethylene (PTFE) surface, and dried at 37° C.

d) Measurement of Equilibrium of HA Hydrogel Films:

Dried hydrogel films were initially cut into 4-mm diameter disks and stained by immersion in 1 mg/mL acridine orange H_2O solution for 60 seconds. The films were then washed with H_2O and re-dried on a PTFE surface to give yellow HA hydrogel films. The diameters of the dried film disks (L_d) were measured. The dyed dried film disks were then placed in either 50 mM phosphate buffer, pH 7.0, or in PBS buffer and allowed to equilibrate at 37° C for 24 hours. Diameters of the swollen hydrogen disks were then again measured under a microscope to determine L_s. The equilibrium swelling ratio (SW) was defined as the weight of absorbed water per weight of dried disk and was calculated using the formula $SW = (L_s/L_d)$.

e) Swelling Kinetics

Swelling was quantified by using the cationic dye acridine orange to stain the hydrogel film in order to visualize the diameter size change during swelling under a microscope. The average equilibrium SW of the hydrogel film in PBS at 37 C was 7.11+/- 0.56. The equilibrium SW in 50 mM pH 7.0 phosphate buffer at 37 C was 7.83 +/- 1.75. No significant difference was observed among the equilibrium SW values in the different buffers, indicating the SW was largely unaffected by the changes in ionic strength. The HA hydrogel films swelled rapidly and reached equilibrium within 100 seconds. The rate of swelling was independent of the buffer composition.

6. Example 6. Hyaluronic Acid Hydrogel Film Preparation with Drug-Loading

a) Materials

Hydrocortisone, dexamethasone, indomethacin, gentamycin, pilocarpine and diclofenac sodium were all obtained from Sigma Chemical Co. (St. Louis, MO) and used as received.

b) Procedure

Hydrocortisone, dexamethasone and indomethacin were all dissolved in ethanol at a concentration of 10 mg/ml. Pilocarpine and diclofenac sodium was dissolved in water at a concentration of 10 mg/ml. An aliquot of each drug was mixed with Solution A, and the *in situ* polymerization method was followed to form a drug-containing hydrogel film. Hydrogel films had drug loading of 5.0% weight percentage relative to their dry macromolecular components. All drug-loaded hydrogel films were obtained by agitating for 24 hours and dried slowly at 37° C.

To prepare a hydrogel film that contained gentamycin, a 10 mg/mL solution was again prepared which was added dropwise with agitation after Solution A was crosslinked by Solution B for 5 minutes. The film was then continuously agitated for 24 hours while the hydrogel film solidified and a film was obtained by drying as above.

c) Results

The release rate of the drugs from the HA hydrogel films was in direct correlation to the hydrophobicity of the drugs. The hydrophobic drugs prednisone and dexamethasone demonstrated prolonged, sustained release, while the more hydrophilic drugs, such as pilocarpine, hydrocortisone, prednisolone, cortisone, diclofenac sodium, indomethacin, 6∝-methyl-prednisolone and corticosterone, demonstrated a rapid release. The rapidly released drugs demonstrated almost complete release from the hydrogel film in 10 minutes following first-order kinetics, consistent with diffusion from the gel during hydration and concomitant swelling of the hydrogel film. In contrast, slow first-order release kinetics was observed for dexamethasone and prednisone. Dexamethasone showed sustained release for 1 hour and prednisone showed sustained release for almost 24 hours.

7. Example 7. Acceleration of Wound Healing

a) Mouse Model

1 cm diameter wounds were created on the back of Balb-c mice. Both the epidermal and dermal layers were removed. The wounds were then dressed with either Biobrane® (control), or ethylene oxide sterilized hyaluronic acid and chondroitin sulfate hydrogel films. Wounds were then bandaged and allowed to heal. Mice were sacrificed after five days and all wound sites excised. Tissue

samples were analyzed histologically. Each group (control, hyaluronic acid hydrogel film, chondroitin sulfate hydrogel film) contained 3 mice. The assay was repeated a total of four times.

Significant re-epithelialization occurred in the wound sites covered with either the hyaluronic acid or chondroitin sulfate hydrogel films, whereas the wound sites covered with the commercially available wound dressing material, Biobrane®, merely began to start the re-epithelialization process of wound healing. The wound covered with the hyaluronic acid hydrogel film had a significantly smaller unhealed wound area than the control group and demonstrated new, thick, several cell-layered, epidermal growth covering the wound area. Additionally, newly-grown non-regulated dermal tissue is filling in underneath the new epidermis. The wound covered with a chondroitin-sulfate hydrogel film had a newly-grown epidermis layer covering the wound site and the non-regulated dermal tissue was filling in underneath the new epidermis. In contrast, the wound covered with Biobrane® demonstrated no epidermal coverage on the surface of the wound site.

Glycosaminoglycan (GAG) based hydrogel films were developed and evaluated for use as wound dressings. Hyaluronic acid (HA) and chondroitin sulfate (CS) were first converted to the adipic dihydrazide derivative (ADH) and then crosslinked with the macromolecular homobifunctional reagent poly(ethylene glycol) propiondialdehyde (PEG-diald) to give a polymer network. These biocompatible materials crosslinked and gelled in minutes. After gelation, a solvent-casting method was used to obtain a GAG hydrogel film. The wound healing aspects of these materials were evaluated in a mouse model. Full thickness wounds created on the dorsal side of a balb/c mouse and were dressed with a GAG film+Tegaderm™ or Tegaderm™ alone. Significant differences in re-epithelialization were found on days 5 ($p<0.001$) and 7 ($p<0.05$) for those wounds treated with a GAG film+Tegaderm™ versus those treated with Tegaderm™ alone. However, no difference was found for wound contraction or inflammatory response.

Additionally, wounds treated with a GAG film had more dermal collagen regeneration and organization by day 10. It is believed that these GAG films provided a highly hydrated, pericellular environment in which assembly of other matrix components, presentation of growth and differentiation factors, and cell migration could readily occur.

(1) Experimental Methods

(a) Materials

Fermentation-derived hyaluronan (HA, sodium salt, MW = $1.5x10^6$) was provided by Clear Solutions Biotech, Inc. (Stony Brook, NY). 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDCI) and adipic dihydrazide (ADH) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chondroitin sulfate C was obtained from Sigma (St. Louis, MO., USA). Poly(ethylene glycol)-propiondialdehyde (PEG-diald) (M_W = 3400) was purchased from Shearwater Polymers, Inc. (Huntsville, AL). Balb/c mice were purchased from Charles River Laboratories Wilmington, MA. Isoflurane was acquired from Abbot Laboratories North Chicago, IL. Tegaderm™ was obtained from 3M Health Care (St. Paul, MN). Curity® Non-Adhering Dressings and Curity® Sheer Bandages were purchased from Kendall Company (Mansfield, MA), and 9 mm Autoclips® were purchased from Becton Dickinson (Sparks, MD).

(b) Analytical instrumentation

Proton NMR spectral data were obtained using a NR/200 FT NMR spectrometer at 200 MHz (IBM Instruments, Inc.). Gel permeation chromatography (GPC) analysis was performed using the following system: Waters 515 HPLC pump, Waters 410 differential refractometer, Waters™ 486 tunable absorbance detector, Ultrahydrogel 250 and 2000 columns (7.8 mm ID x 130 cm) (Milford, MA). Eluent was 150 mM phosphate buffer (pH 6.5) / MeOH = 80:20 (v/v) and the flow rate was 0.5 mL/min. The system was calibrated with standard HA samples provided by Dr.

U. Wik (Pharmacia, Uppsala, Sweden).

(c) Preparation of GAG-ADH

HA-ADH was made using methods previously reported (Luo, Y., Kirker, K. R., and Prestwich, G. D. (2000) Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release* 69, 169-184). Briefly, HA was dissolved in H_2O at 5 mg/mL. 5 molar equivalents of ADH, and 3 molar equivalents of EDCI were added in solid form, while maintaining the pH at 4.75, with the addition of 1.0 N HCl. The reaction was stopped by raising the pH of reaction mixture to 7.0, and the reaction mixture was dialyzed exhaustively. The solution was then centrifuged, and the supernatant was lyophilized. The purity of HA-ADH was measured by GPC, and the degree of substitution by ADH was determined by ^{1}H-NMR.

CS-ADH was obtained using the same procedures, except CS was dissolved in H_2O at 25 mg/mL and 5 and 2 molar equivalents of ADH and EDCI were used respectively.

(d) Preparation of GAG-PEG hydrogel films

GAG-PEG films were made as described (Luo, Y., Kirker, K. R., and Prestwich, G. D. (2000) Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release* 69, 169-184). GAG-ADH was dissolved in H_2O (5 mg/mL for HA-ADH and 25 mg/mL for CS-ADH) to form Solution A. PEG-diald was dissolved in H_2O at a concentration of 50 mg/mL (Solution B). Volumes of Solutions A and B were added to a small polystyrene dish to give desired equivalents of aldehyde and hydrazide functionalities, and the solutions were mixed with gentle swirling. A hydrogel formed within 60 sec. The mixture was agitated on an orbital platform for an additional 24 hr to obtain a solid, uniform hydrogel. Hydrogels were stored in an open dish overnight at 37°C to allow

solvent evaporation and thus provide a flexible, hydratable HA hydrogel film.

(e) In vivo wound healing

The wound healing characteristics of the GAG films were evaluated using a mouse model. All assays were completed with the approval of the University of Utah's Institutional Animal Care and Use Committee. To begin, a male balb/c mouse, weighing approximately 25 g was anesthetized with 2.5% isoflurane using a VetEquip inhalation anesthesia system. The surgical area was shaved with an electric razor, the mouse was strapped to a surgical board, and additional anesthesia was provided via a nose cone.

After a deep surgical plain had been reached, a wound, approximately 1.0-cm in diameter, was created on the dorsal side of the mouse using curve blade surgical scissors. Both the epidermal and dermal layers were removed, creating a full thickness wound with minimal bleeding. Next, four, individual diameters of the wound site were marked and measured using digital calipers and averaged to determine the original wound diameter and area. The wound was then dressed with one of three dressings: (1) Tegaderm™ (control); (2) ethylene oxide sterilized HA film and Tegaderm™; or (3) ethylene oxide sterilized CS film and Tegaderm™. GAG films were cut into 13-mm in diameter circular sheets that were rehydrated with sterile normal saline before use. The Tegaderm™ was cut into 2-cm square pieces, pieces large enough to cover the entire wound and the surrounding area. The dressed wound was then covered with a Curity® Non-Adhering Dressing, bandaged with a Curity® Sheer Bandage, sealed with two 9 mm Autoclips®, and allowed to heal. The surgery was repeated multiple times to give a sample size of six mice per treatment per time point (described below).

On day 3, 5, 7, or 10 post-surgery, the mouse was anesthetized with isoflurane and sacrificed by cervical dislocation. The four wound diameters were measured again to determine the new wound area. The wound site was excised, and

the tissue was processed for histological evaluation. Any wound that had been exposed to air, because the mouse had chewed off its bandage, was not used for analysis. The wound size measurements taken at the time of surgery and at the time of biopsy were used to calculate the percent wound contraction, using equation 1.

$$\% \text{ wound contraction} = (A_o - A_t)/A_o \times 100 \quad (1)$$

where: A_o = original wound area and A_t = area of wound at time of biopsy

(f) Histology

Excised wound sites were formalin fixed, processed, and embedded in paraffin. 3-5 μm thick sections were stained with hematoxyline and eosin (H&E) and Masson's Trichrome stain. For each wound, six on-edge sections spanning the diameter of the original wound site were taken and examined by conventional microscopy.

Histological endpoints included a quantitative measurement of the percent re-epithelialization, a qualitative measurement of the inflammatory response, and the presence of new vasculature. The un-epithelialized wound diameter was measured using an eyepiece micrometer. This measurement, together with the original wound diameter was used in equation 2 to determine the percent re-epithelialization. The average of all six sections from each wound site was calculated and determined to be the average of the percent re-epithelialization for that wound.

$$\% \text{ re-epithelialization} = (D_o - D_B)/D_o \times 100 \quad (2)$$

where: D_o = original wound diameter and D_B = diameter of wound at time of biopsy

The inflammatory response was graded on a qualitative scale from 0-3 (0- no sign of inflammation, 1-minimal inflammation, 2-moderate inflammation, and 3-strong presence of inflammatory cells). The presence of new vascular tissue in the

newly regenerated dermis indicated angiogenesis.

(g) Statistical Analysis

All data is presented as the mean ± standard deviation, and all statistical analysis for significance was determined using ANOVA with $\alpha = 0.05$ and a p • 0.05 (using StatView) being considered as significant.

(2) Results

(a) Preparation of GAG-ADH

Separately, purified HA-ADH and CS-ADH were dissolved in D_2O and analyzed using H^1NMR. The degree of substitution was determined by integration of the ADH methylene signals using the *N*-acetyl methyl resonances (δ = 1.95~2.00 ppm) as an internal standard (Pouyani, T., Harbison, G. S., and Prestwich, G. D. (1994) Novel hydrogels of hyaluronic acid: synthesis, surface morphology, and solid-state NMR. J Am Chem Soc 116, 7515-7522; Pouyani, T., and Prestwich, G. D. (1994) Functionalized derivatives of hyaluronic acid oligosaccharides - drug carriers and novel biomaterials. Bioconjugate Chemistry 5, 339-347.). The degree of ADH substitution of the HA-ADH used in this Example was 55%, based on the number of available glucuronates modified, while the CS-ADH was 88% modified.

(b) Preparation of GAG-PEG hydrogel films

The GAG-ADH was crosslinked with PEG-diald, which produced a bis-hydrazone functionality as the covalent crosslink (Figure 1). Hydrogels were produced when the crosslinking agent (PEG-diald) was used in a molar ratio of 0.25, 0.5, and 1 relative to ADH. The hydrogel began to form within 60 sec after mixing of the GAG-ADH and PEG-diald solutions, but was agitated for 24 hrs. to assure the formation of a solid, uniform hydrogel. Evaporation of the solvent provided flexible, hydratable GAG hydrogel film. The HA-ADH films used in these investigations were 100% crosslinked (aldehyde:ADH ratio of 1:1), while CS-ADH

films were 25% crosslinked to keep the quantity of PEG in both film types consistent.

(c) In vivo wound healing

Wound contraction is a healing process whereby the edges of the wound pull inwards to reduce the overall wound area. During healing, wound fibroblasts begin to assume a myofibroblast phenotype characterized by large bundles of actin containing microfilaments and the establishment of cell-cell and cell-matrix linkages (Clark, R. A. F. Wound repair: Overview and general considerations. In The Molecular and Cellular Biology of Wound Repair; R. A. F. Clark, Ed.; Plenum Press: New York, 1996; pp 3-50). The fibroblasts link to extracellular fibronectin and collagen and to each other through adherens junctions. Collagen bundles at the wound edge and the underlying dermis crosslink to form a collagen network. These cell-cell, cell-matrix, and matrix-matrix links provide a network through which the traction of the fibroblasts can be transmitted across the wound, thereby pulling the wound edges inward.

Re-epithelialization is the process through which new cutaneous tissue covers the wound defect. This process requires the uninjured keratinocytes along the wound edges to migrate laterally to cover the wound bed. Both wound contraction (Fang, C.-H., Robb, E. C., Yu, G.-S., Alexander, J. W., and Warden, G. D. (1990) Observations on stability and contraction of composite skin grafts: Xenodermis or allodermis with an isograft overlay. Journal of Burn Care and Rehabilitation 11, 538-542; Joseph, H. L., Roisen, F. J., Anderson, G. L., Barker, J. H., Weiner, L. J., and Tobin, G. R. (1997) Inhibition of wound contraction with locally injected lathyrogenic drugs. American Journal of Surgery 174, 347-350; Kaufman, T., Kalderon, N., Ullmann, Y., and Berger, J. (1988) Aloe vera gel hindered wound healing of experimental second-degree burns: A quantitative controlled study. Journal of Burn Care and Rehabilitation 9, 156-159; Noormohamed, S. E., and Ray, T. (1998) Effect of 'Compound R' on thermal burn and full-depth wound contracture

in fuzzy rats. Journal of Burn Care and Rehabilitation 19, 213-215; Rennekampff, H. O., Kiessig, V., Griffey, S., Greenleaf, G., and Hansbrough, J. F. (1997) Acellular human dermis promotes cultured keratinocyte engraftment. Journal of Burn Care & Rehabilitation 18, 535-544) and re-epithelialization (Chvapil, M., Gaines, J. A., and Gilman, T. (1988) Lanolin and epidermal growth factor in healing of partial thickness pig wounds. Journal of Burn Care and Rehabilitation 9, 279-284; Davis, S. C., Badiavas, E., Rendon-Pellerano, M. I., and Pardo, R. J. (1999) Histological comparison of postoperative wound care regimens for laser resurfacing in a porcine model. Dermatol Surg 25, 387-393; Singer, A. J., Berrutti, L., Jr., H. C. T., and McClain, S. A. (1999) Octylcyanoacrylate for the treatment of partial-thickness burns in swine: A randomized, controlled experiment. Academic Emergency Medicine 6, 688-692; and Singer, A. J., Berrutti, L., and McClain, S. A. (1999) Comparative trial of octyl-cyanoacrylate and silver sulfadiazine for the treatment of full-thickness skin wounds. Wound Repair and Regeneration 7, 356-361) have been used as methods of monitoring wound closure and healing. In this paper, wound contraction was monitored by measuring the area within the wound's full-thickness margins, and re-epithelialization was determined by measuring the advancing epithelium.

Figures 2 and 3 show the results for both wound contraction and re-epithelialization, respectively. No significant difference in wound contraction was observed between either of the two assay groups (HA film or CS film treated wounds) and the control group (Tegaderm™ only) for any time point. On the other hand by day 5, wounds treated with CS film had significantly ($p<0.001$) more epithelial tissue than the controls. Additionally by day 7 post-surgery, wounds treated with either GAG film hand significantly more ($p<0.05$) epithelial coverage.

Other histological differences between the groups are listed in Table 1. (histological samples were illustrated by Masson's Trichrome stained sections of wounds 5 and 10 days post surgery at 10x magnification. Various samples were

looked at for example, (a) Wound dressed with HA film+Tegaderm™; (b) Wound dressed with CS film+Tegaderm™; (c) Wound dressed with Tegaderm™ only; (d) Tissue peripheral to wound bed as an example of normal skin. A-adipose, D-dermis, E-epidermis, F-HA film, M-muscle. At day 5, heavy wound exudate was noticed on the HA film dressed wound, the early appearance of new epidermis was observed on the CS dressed wound, and neither was observed on the Tegaderm™ only wound. At day 10, there was heavy dermal collagen regeneration and reorganization in the HA and CS film dressed wounds and the lack of in the Tegaderm™ only wound.

Treatment	Percent of mice completely re-epithelialized	Presence of new blood vessels (+/-)	Inflammatory Response		Comments
			Dermis	Muscle	
Day 3					
Tegaderm™	0	-	+++	++	a
HA+Tegaderm™	0	-	+++	++	b
CS+Tegaderm™	0	-	+++	++	c
Day 5					
Tegaderm™	0	-	+++	++	a
HA+Tegaderm™	0	-	+++	+++	b, c
CS+Tegaderm™	33	+	+++	+++	d
Day 7					
Tegaderm™	0	+	+++	++	c
HA+Tegaderm™	33	+	+++	+	b, d
CS+Tegaderm™	67	+	+++	+	d
Day 10					
Tegaderm™	17	+	+++	++	c
HA+Tegaderm™	67	+	+++	++	d
CS+Tegaderm™	83	+	+++	+	d

Table 1: Histological evaluation of wounds for all three assay groups at all time points. Inflammatory response: (+++) strong, (++) moderate, (+) minimal, (-) none Comments: (a) little dermal regeneration, (b) film integrating into wound, (c) moderate dermal rengeration, (d) visual signs of dermal collagen organization

Notable day 3 and 5 differences involve heavy wound exudate. HA film treated wounds were covered with a moist, degrading film, full of wound exudate. Attempts to remove the film resulted in bleeding, indicating that the film was

integrated into the wound bed. Biopsies and histology revealed a thick wound bed, heavily infiltrated with inflammatory cells, primarily polymorphonuclear leukocytes, identified by their lobular nuclei. Little exudate was found on the CS film treated wounds, and the films were easily removed from the wound site. By day 5 there was complete epithelial coverage on 2 of CS film treated wounds plus visual identification of new vasculature and dermal organization was evident in all of the CS film treated wounds. With either GAG treatment, wound edges were identified by the heavy proliferation of the peripheral keratinocytes, which were migrating through the dermis-like space provided by the films. The Tegaderm™ only treated wounds had no wound exudate, little dermal regeneration, and inflammatory response. The Tegaderm™ dressing adhered to the wound bed, and removal resulted in the loss of tissue at the wound site. Therefore, all Tegaderm™ only dressing wounds were histologically processed with the Tegaderm™ still in place.

Seven days after surgery 1 HA film treated wound and 4 CS film treated wounds, while none of the Tegaderm™ only treated wounds were completely re-epithelialized. All the wounds were heavily infiltrated with inflammatory cells, but the inflammation was localized only to the new dermis. On previous days, the underlying muscle had a heavy inflammatory presence.

By day 10 post-surgery the HA films were completely degraded with no film visibly present on the wound bed. Four of the six wounds had completely re-epithelialized and dermal collagen regeneration. Similar results were seen with the CS film treated wounds, except the CS film had not yet degraded. Five of the six wounds had 100% re-epithelialization and the collagen fibrils of the dermis were well organized. On the other hand, results for the control wounds were, more inconsistent, exhibited less re-epithelialization, and little dermal collagen regeneration and organization.

An ideal wound dressing has many attributes. It should protect the wound

from bacterial infection, control evaporative water loss and prevent dehydration, control permeability of oxygen and carbon dioxide, absorb wound exudate, and enhance the healing. Additionally, it should be composed of materials that are non-toxic, non-immunogenic, flexible, durable, and comfortable when worn.

The films described herein have significant advantages to the previously studied materials. They are hydrophilic and absorb wound exudate. They can be used immediately and do not require weeks of preparation. The HA and CS films are biodegradable and non-immunogenic, and they can be used with an occlusive dressing to prevent infection. Additionally, the HA hydrogel films describe here have been shown to possess sustained release capabilities (Luo, Y., Kirker, K. R., and Prestwich, G. D. (2000) Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release* 69, 169-184) that may prove useful for delivering therapeutic agents at wound sites.

The mouse *in vivo* wound healing assays indicate that significant healing occurred in wounds treated with a GAG hydrogel film through the re-epithelialization process. Additionally, the underlying dermal tissue appeared to regenerate and reorganize more rapidly than those wounds treated with Tegaderm™ alone. The control, Tegaderm™, consists of a thin polyurethane membrane coated with a layer of an acrylic adhesive. The dressing, which is permeable to both water vapor and oxygen, is impermeable to microorganisms. Clinically, Tegaderm™ is used in the treatment of minor burns, pressure areas, donor sites, post-operative wounds, and a variety of minor injuries including abrasions, and lacerations.

Combining the use of GAG films with Tegaderm™ created at wound dressing that protected the wound from bacterial infection, controlled evaporative water loss and prevent dehydration, controlled permeability of oxygen and carbon dioxide, absorbed wound exudate, and enhanced the healing. It is probable that the HA films promote cell movement in early granulation tissue as HA has been found

to do in embryogenesis and morphogenesis (Toole, B. P. (1997) Hyaluronan in morphogenesis. *J Intern Med* 242, 35-40).

Histological results from those wounds treated with a CS film show more dermal collagen regeneration and organization than those treated with a HA film or Tegaderm™. Finally, other GAG molecules like heparin and heparan sulfates have been found to bind, hold, and act as a repository for a large group of cytokines (Gallo, R. L., and Bernfield, M. Proteoglycan and their role in wound repair. In The Molecular and Cellular Biology of Wound Repair; R. A. F. Clark, Ed.; Plenum Press: New York, 1996; pp 475-492.).

Wound exudate mixed with degrading film was clearly visible upon gross and histological examination of the HA film treated wounds.

b) Pig Model

Assays similar to those conducted in mouse were also conducted in Pig with similar results.

(1) Experimental Methods

(a) Materials

Fermentation-derived hyaluronan (HA, sodium salt, MW = $1.5x10^6$) was provided by Clear Solutions Biotech, Inc. (Stony Brook, NY). 1-Ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDCI) and adipic dihydrazide (ADH) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Chondroitin sulfate C was obtained from Sigma (St. Louis, MO., USA). Poly(ethylene glycol)-propiondialdehyde (PEG-diald) (M_W = 3400) was purchased from Shearwater Polymers, Inc. (Huntsville, AL). Female pigs were purchased from Arnold's Hog Supply (Lehi, Ut). Tegaderm™ and VetWrap were obtained from 3M Animal Care Products (St. Paul, MN). Skin markers were purchased from DeRoyal

(Powell, TN). Betasept® was acquired from Purdue Frederick (Norwalk, CT). Lap Sponges were obtained from Medical Action Industries (Ashville, NC). AutoSuture™ skin staples were purchased form United States Surgical Corporation (Norwalk, CT).

(b) Analytical instrumentation

Proton NMR spectral data were obtained using a NR/200 FT NMR spectrometer at 200 MHz (IBM Instruments, Inc.). Gel permeation chromatography (GPC) analysis was performed using the following system: Waters 515 HPLC pump, Waters 410 differential refractometer, Waters™ 486 tunable absorbance detector, Ultrahydrogel 250 and 2000 columns (7.8 mm ID x 130 cm) (Milford, MA). Eluent was 150 mM phosphate buffer (pH 6.5) / MeOH = 80:20 (v/v) and the flow rate was 0.5 mL/min. The system was calibrated with standard HA samples provided by Dr. U. Wik (Pharmacia, Uppsala, Sweden).

(c) Preparation of GAG-ADH

HA-ADH was made using slightly modified methods from those previously reported (Luo, Y., Kirker, K. R., and Prestwich, G. D. (2000) Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release* 69, 169-184). First, high molecular weight HA (MW=1.5x10^6) was degraded using acid degradation. 20 g of HA was dissolved in 2.5 L H_2O by vortexing at 150 rpm at 37°C. After 3 hrs, the solution was moved to a mechanical stirrer and the pH of the solution was adjusted to 0.6-0.7 through the addition of concentrated HCl. The mixture was allowed to stir for an additional 24 hrs. Afterwards the pH of the solution was raised to 7.0 through the addition of 0.1 N NaOH, and the solution was transferred to the pre-washed dialysis tubing (MWCO=3,500) and dialyzed exhaustively against H_2O. The molecular weight of the resulting low molecular weight HA (LMWHA) was determined by GPC.

LMWHA solution was then used to prepare HA-ADH. 10 molar equivalents

of ADH, and 2 molar equivalents of EDCI were added in solid form, while maintaining the pH at 4.75, with the addition of 1.0 N HCl. Raising the pH of reaction mixture to 7.0 stopped the reaction, and the reaction mixture was dialyzed thoroughly. The solution was then centrifuged, and the supernatant was lyophilized. The purity of HA-ADH was measured by GPC, and the degree of substitution by ADH was determined by ^{1}H-NMR. CS-ADH was obtained using the same procedures, except no adjustments were made to its molecular weight, it was dissolved in H_2O at 25 mg/mL, and 5 and 2 molar equivalents of ADH and EDCI were used respectively.

(d) Preparation of GAG-PEG hydrogel films

GAG-PEG films were produced using methods similar to those previously reported (Luo, Y., Kirker, K. R., and Prestwich, G. D. (2000) Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release* 69, 169-184). 2.5 g of GAG-ADH was dissolved in 100 mL of nanopure H_2O to form Solution A. The desired equivalent of PEG-diald was dissolved in H_2O at a concentration of 25 mg/mL (Solution B). Both solutions were cooled to 4°C, added to a 25.4-cm2 polystyrene dish, and mixed with gentle vortexing. A hydrogel formed within 5 minutes. The mixture was agitated on an orbital platform for an additional 24 hr to obtain a solid, uniform hydrogel. Hydrogel was then moved to a 37°C incubator and stored overnight to allow solvent evaporation. The resulting film was then cut into 5 x 5 cm^2 pieces. Each piece was sterilized with ethylene oxide gas and stored separately at room temperature until use.

(e) In vivo wound healing

The wound healing characteristics of the GAG films were evaluated using a pig model (Figure 4). All assays were completed with the approval of the University of Utah's Institutional Animal Care and Use Committee. To begin, a female pig,

weighing approximately 40 kg was anesthetized with TKX (1-2 cc/50 kg) and Atropine (0.12-0.15 mg/kg) and intubated. The surgical area was shaved with an electric razor and prepped with iodine solution. Additional anesthesia (1-2% isoflurane), heart rate, and respiration were monitored with a surgical anesthesia machine.

After a deep surgical plain had been reached, a skin marker was used to outline the location of the 8 5 x 5 cm^2 desired surgical sites on the dorsal side of the ing the spine as the sagittal plane, 4 sites were located on each side of the pig wo wounds located medially and two laterally. Next, each intended site was lub ed with Betasept™, and a partial thickness wound was inflicted with a adgett Electro-Dermatome from Padgett Instruments Kansas City, MO, creating wounds that were approximately 5 x 5 x 0.03 cm^3. After haemostasis was reached, the exact wound dimensions were measured and the wounds were dressed. The four wounds on one half of the pig were dressed with a GAG film plus Tegaderm™, while the other half were dressed with Tegaderm™ alone. All dressed wounds were then covered with 4 Lap Sponges, wrapped with Vetrap™ and a surgical stocking, and secured with skin staples. The surgery was repeated multiple times to give a sample size of three pigs per treatment per time point (described below).

On day 3, 5, or 7 post-surgery, the pig was anesthetized with TKX (1-2 cc/50 kg) and Atropine (0.12-0.15 mg/kg) and sacrificed with Beuthanasia (20 cc). Each wound was excised in its entirety and analyzed histologically.

(f) Histology

Each excised wound was divided into four equal pieces, and each piece was formalin fixed, processed, and embedded in paraffin (Figure 5). One 3-5 μm thick on edge section from each piece was stained with Masson's Trichrome stained. For each wound, one section from each piece of wound was examined, for a total of three sections per wound.

Histological endpoints included a quantitative measurement of the percent re-epithelialization, a qualitative measurement of the inflammatory response, and a quantitative measurement of the dermal regeneration. The un-epithelialized wound distance was measured using an eyepiece micrometer. This measurement, together with the original wound size was used in equation 1 to determine the percent re-epithelialization. The average of all three sections from each wound site was calculated and determined to be the average of the percent re-epithelialization for that wound. Next, the results from each wound were averaged for the percent re-epithelialization for that particular pig.

$$\% \text{ re-epithelialization} = (D_o - D_B)/D_o \times 100 \quad (1)$$

where: D_o = original wound length and D_B = length of wound at time of biopsy

The inflammatory response was graded on a qualitative scale from 0-3 (0- no sign of inflammation, 1-minimal inflammation, 2-moderate inflammation, and 3-strong presence of inflammatory cells). Dermal regeneration was determined using computer densitometry. For each tissue section, the density of dermal collagen (stained blue from the Masson's Trichrome stain) peripheral to the wound bed determined. Next, the density of new collagen in the wound bed was determined, and the ratio of densities was calculated and correlated to dermal regeneration (Figure 6).

(g) Statistical Analysis

All data is presented as the mean ± standard deviation, and all statistical analysis for significance was determined using ANOVA with $\alpha = 0.05$ and a $p \bullet 0.05$ (using StatView) being considered as significant.

(2) Results

(a) Preparation of GAG-ADH

Separately, purified HA-ADH and CS-ADH were dissolved in D_2O and analyzed using H^1NMR. The degree of substitution was determined by integration of the ADH methylene signals using the *N*-acetyl methyl resonances (δ = 1.95~2.00 ppm) as an internal standard (Pouyani, T., Harbison, G. S., and Prestwich, G. D. (1994) Novel hydrogels of hyaluronic acid: synthesis, surface morphology, and solid-state NMR. *J Am Chem Soc* 116, 7515-7522). The degree of ADH substitution of the LMWHA-ADH used in this Example was 63%, based on the number of available glucuronates modified, while the CS-ADH was 83% modified.

(b) Preparation of GAG-PEG hydrogel films

The GAG-ADH was crosslinked with PEG-diald, which produced a bis-hydrazone functionality as the covalent crosslink. The hydrogel began to form within 5 minutes after mixing of the GAG-ADH and PEG-diald solutions, but was agitated for 24 hrs. to assure the formation of a solid, uniform hydrogel. Evaporation of the solvent provided flexible, hydratable GAG hydrogel film. The GAG-ADH films used in these investigations were 50% crosslinked (aldehyde:ADH ratio of 0.5:1).

(c) In vivo wound healing

Re-epithelialization is the process through which new cutaneous tissue covers the wound defect. This process requires the uninjured keratinocytes along the wound edges and the lining the hair follicles to migrate, covering the wound bed. Monitoring such epithelial migration has been used as methods of monitoring wound closure and healing (Chvapil, M., Gaines, J. A., and Gilman, T. (1988) Lanolin and epidermal growth factor in healing of partial thickness pig wounds. *Journal of Burn Care and Rehabilitation* 9, 279-284; Davis, S. C., Badiavas, E., Rendon-Pellerano, M. I., and Pardo, R. J. (1999) Histological comparison of postoperative wound care

regimens for laser resurfacing in a porcine model. *Dermatol Surg* 25, 387-393; Singer, A. J., Berrutti, L., Jr., H. C. T., and McClain, S. A. (1999) Octylcyanoacrylate for the treatment of partial-thickness burns in swine: A randomized, controlled experiment. *Academic Emergency Medicine* 6, 688-692; and Singer, A. J., Berrutti, L., and McClain, S. A. (1999) Comparative trial of octyl-cyanoacrylate and silver sulfadiazine for the treatment of full-thickness skin wounds. *Wound Repair and Regeneration* 7, 356-361.). Wound healing was quantified by the percent re-epithelialization of partial thickness wounds created on the dorsal side of a pig.

On day 3 post-surgery, gross evaluation of the wounds revealed a thick, exudate covering the GAG film treated wounds (histology was performed at 3, 5, and 7 days post surgery. Typically sections for the following were examined; (a) Wound treated with an HA film+Tegaderm™ on day 3; (b) Wound treated with Tegaderm™ only on day 3; (c) Wound treated with CS film+Tegaderm™ on day 5; (d) Wound treated with Tegaderm™ only on day 5; (e) Wound treated with HA film+Tegaderm™ on day 7; and (f) Wound treated with Tegaderm™ only on day 7. (D-dermis, E-epidermis, F-HA film and exudate, and H-hair follicle.) There was exudate on the day 3 and 5 GAG treated wounds.).

The coating was actually degrading GAG film impregnated with wound exudate. Attempts to remove the exudate/film resulted in bleeding, indicating that the film was integrated into the wound bed. On the other hand, Tegaderm™ only treated wounds had no exudate covering and looked dehydrated. Histology revealed that the HA film dressed wounds exhibited significantly more re-epithelialization than the control group (Tegaderm™ only) (Figure 7). Epithelial cells from wound edges and hair follicles proliferated and migrated to cover the wound (Typically this type of date was obtained byanalyzing Masson's Trichrome stained sections of wounds 3 days post surgery at 4x magnification. Typically the following sections were analyzed: (a) Wound dressed with HA film+Tegaderm™; (b) Wound dressed

with Tegaderm™ only. Typically heavy excessive proliferation of epithelial cells migrating from the hair follicles was observed on the HA film treated wound.).

The gross evaluations for day 5 post-surgery were similar to those of day 3.

By day 7, the majority of all wounds were completely healed. The GAG films were degraded into small fragments and were easily washed off with normal saline. The Tegaderm™ only dressed wound also appeared completely healed. Histological evaluation confirmed these results (Figure 7).

These preliminary results indicate that the presence of a GAG film helps to accelerate the re-epithelialization process. By day 3 epithelial cells are proliferating and migrating from both the wound edges and hair follicles to cover the wound bed, with significantly more coverage found than in the HA+Tegaderm™ dressed wounds. By day 7 both treatment groups are completely healed.

8. Example 8. Mechanical testing of GAG films

a) Methods

(1) Preparation of GAG-PEG hydrogel films

GAG-PEG films were made as described (Luo, Y., Kirker, K. R., and Prestwich, G. D. (2000) Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery. *Journal of Controlled Release* 69, 169-184). GAG-ADH was dissolved in H_2O (5 mg/mL for HA-ADH and 25 mg/mL for CS-ADH) to form Solution A. PEG-diald was dissolved in H_2O at a concentration of 50 mg/mL (Solution B). Volumes of Solutions A and B were added to a small polystyrene dish to give desired equivalents of aldehyde and hydrazide functionalities, and the solutions were mixed with gentle swirling. A hydrogel formed within 60 sec. The mixture was agitated on an orbital platform for an additional 24 hr to obtain a solid,

uniform hydrogel. Hydrogels were stored in an open dish overnight at 37°C to allow solvent evaporation and thus provide a flexible, hydratable HA hydrogel film.

(2) Mechanical testing

The mechanical testing of the GAG films were completed using methods outlined in ASTM D882-95a. Briefly, the film was first cut into 70 x 7 x 0.045 mm^3 sized samples with exact measurements taken at the time of testing. The film was clamped into the bottom and top grips of a Model TMS (serial #136) Instron (Instron, Canton, Mass.) with an Instron 10-lb maximum-capacity load cell (5-lb. load used for testing). The samples were then strained at a cross-head speed of 0.8 in/min until failure, and the load supported by the film was recorded on a strip-chart recorder. The modulus, tensile stress, and % elongation at failure were then calculated.

For testing of the swollen mechanical properties similar procedures were followed. A dry film was clamped into position on the Instron. Next, the film was sprayed with PBS for 30 seconds and allowed to swell and equilibrate. Afterwards, using a 2 lb. load, the film was strained at a cross-head speed of 0.8 in/min. Again, the load supported by the film was recorded on a strip-chart recorder, and the modulus, tensile stress, and % elongation at failure were calculated.

For comparison, human skin was also tested. The tissue was thawed and cut into 50 mm x 7 mm sized samples. Human epidermis samples were tested using the 2-lb. load and a crosshead speed of 0.8 in/min.

b) Results

(1) Preparation of GAG-PEG hydrogel films

The GAG-ADH was crosslinked with PEG-diald, which produced a bis-hydrazone functionality as the covalent crosslink (Figure 1B). Hydrogels were

produced when the crosslinking agent (PEG-diald) was used in a molar ratio of 0.25, 0.5, and 1 relative to ADH. The hydrogel began to form within 60 sec after mixing of the GAG-ADH and PEG-diald solutions, but was agitated for 24 hrs. to assure the formation of a solid, uniform hydrogel. Evaporation of the solvent provided flexible, hydratable GAG hydrogel film. The GAG-ADH films used in these investigations were 100 and 50% crosslinked (aldehyde:ADH ratio of 1:1 and 0.5:1).

(2) Mechanical Testing

The mechanical testing results are listed in Tables 2 and 3. Briefly, the modulus of elasticity of the HA film increases with increasing crosslinking, while the elongation decreases, as expected. The higher crosslinking makes the film more rigid, preventing elastic deformation of the material. There was no difference in tensile strength for the two types of crosslinked HA films. However, the CS films showed the opposite results. The 100% crosslinked films have lower modulus and tensile strength than the 50% crosslinked films, with no significant differences in the elongation at failure. It is possible that when making the 100% crosslinked CS films all the PEG-diald did not react, resulting in a less crosslinked film with less than expected mechanical results.

When swollen with PBS, all films have considerably less strength and elasticity, yet similar elongation values compared to the dry films. Values for swollen films are very similar to human epidermis. Again, the results from the swollen HA films are the opposite than expected. The 100% crosslinked films have lower modulus and tensile strength than the 50% crosslinked films. Therefore, it is speculated that the mechanical properties of the films can be manipulated and tailored by changing the % crosslinking.

Table 2: Mechanical properties of dry GAG films (avg. ± s.d., n=9)

	Modulus of Elasticity (MPa)	Tensile Stress (MPa)	% Elongation at Failure
HA Films			
100% crosslinked	790 ± 64	20 ± 4	12 ± 5
50% crosslinked	400 ± 130	21 ± 4	49 ± 6
CS Films			
100% crosslinked	170 ± 28	12 ± 3	46 ± 14
50% crosslinked	420 ± 110	28 ± 4	35 ± 9

Table 3: Mechanical properties of swollen GAG films (avg. ± s.d., n=9)

	Modulus of Elasticity (MPa)	Tensile Stress (MPa)	% Elongation at Failure
HA Films			
100% crosslinked	1.0 ± 0.3	0.20 ± 0.06	15 ± 6
50% crosslinked	2.6 ± 0.6	0.82 ± 0.23	30 ± 10
CS Films			
50% crosslinked	1.4 ± 0.2	0.16 ± 0.04	21 ± 14
Epidermis	0.96 ± 0.52	0.28 ± 0.04	28 ± 11

9. Example 9. Microvasculature growth stimulated by HA hydrogels

HA-ADH hydrogels were formed by cross-linking with PEG-dialdehyde (As discussed in Example 2), then dried to a film form by sitting at 37 °C for 24 hours. All steps were carried out under clean conditions. Three cases of films were formed:

1) a film containing HA-ADH only

2) a film containing HA-ADH into which 2μg VEGF had been mixed prior to cross-linking; both of these films were used without further sterilization.

3) and a film containing HA-ADH + VEGF that was sterilized by exposure to ethylene oxide after drying.

a) Experimental

5mm x 5mm square samples were cut from the films and surgically implanted in a small pocket that was formed in a mouse ear immediately beneath its skin. These implants contained 1.0 mg of cross-linked HA, and the VEGF cases also contained 50ng of VEGF. Along with the animals receiving implants, a sham surgery was also performed in which a pocket was formed, but no implant was placed in it. Three animals were treated for each case.

At 14 days post-surgery, the animals were sacrificed and both the implanted and contralateral ears were retrieved. The ears were fixed in formalin, sectioned for microscopy and stained with hematoxylin and eosin. The numbers of microvessels in 10 randomly chosen 100 sq. micrometer patches from each ear, surgical and contralateral, were then counted by two observers.

Figure 8 shows microvessel density for each case, averaged over the three animals. All differences were statistically significant by one-way ANOVA, with $p < 0.005$, except for the sterilized vs. non-sterilized HA + VEGF comparison which had $p = 0.06$. Although the pocket formation merely by itself elicited notable microvessel growth, since the sham mean (122 microvessels/mm^2) was nearly twice the contralateral mean (63 microvessels/mm^2), nevertheless the presence of the implant dramatically amplified this effect. HA alone produced nearly twice the microvessel density of the sham (222 vs 122 microvessels/mm^2), and HA + VEGF (non-sterile) produced an additional 150% increase (372 vs. 222).

These results demonstrate clearly that (a) in general, HA hydrogels can be highly effective motifs for delivery of endogenous pharmaceutical agents and (b) specifically, HA and the combination of HA with VEGF can dramatically enhance the process of neovasculogenesis in *in vivo* applications. A summary of these results is shown in Figure 8.

10.Example 10. The release of growth factors from GAG films

a) Methods

(1) Preparation of HA film with bFGF

To begin, 2 μg of human recombinant basic Fibroblast Growth Factor (bFGF) (Sigma, St. Louis, MO) was resuspended in 1 ml of 0.1% bovine serum albumin (BSA) in phosphate buffered saline (PBS). Next, 37.5 mg of low molecular weight HA-ADH (LMWHA-ADH) was dissolved in H_2O at 25 mg/mL to form Solution A. The bFGF solution was then mixed into Solution A. 40.07 mg of PEG-diald was dissolved in H_2O at a concentration of 25 mg/mL to form Solution B. Both solutions were cooled to 4°C, added to a 3.5 cm in diameter petri dish, and mixed with gentle vortexing. A hydrogel formed within 5 minutes. The mixture was agitated on an orbital platform for an additional 24 hr to obtain a solid, uniform hydrogel. The hydrogel was then moved to a 37°C incubator and stored overnight to allow solvent evaporation and thus provide a flexible, hydratable HA hydrogel film. The resulting film was cut into 1-cm in diameter disks and stored separately. One half of the disks also received ethylene oxide gas sterilization.

(2) bFGF release

An *in vitro* release assay was used to monitor the release of bFGF from the HA films. To begin, a HA/bFGF disk was placed in a small glass vial. 1 ml of 100 U/ml of HAse in PBS was then added to the vial. The container was then placed on an orbital platform at 37°C and incubated. Periodically, the bathing solution was recovered and replaced with new solution. The recovered solution was stored at –80°C until analyzed. The amount of bFGF in the collected solutions was quantified using the Quantikine® immunoassay by R&D Systems (Minneapolis, MN) and the methods described there in.

(3) Preparation of GAG film with TGF-β

Human recombinant transforming growth factor beta 3 (TGF-β) was incorporated into both HA and CS films. To begin, 1.5 ng of TGF-β (Oncogene Research Products, Boston, MA) was resuspended as directed in sterile 4 mM HCl with 0.1 % BSA. Next, 109.5 mg of GAG-ADH (either LMWHA-ADH or CS-ADH) was dissolved in H_2O at 25 mg/mL to form Solution A. The TGF-β solution was then mixed into Solution A. A desired amount of PEG-diald (approximately 124 mg for either a HA or CS film) was dissolved in H_2O at a concentration of 25 mg/mL to form Solution B. Both solutions were cooled to 4°C, added to a 6 cm in diameter petri dish, and mixed with gentle vortexing. A hydrogel formed within 5 minutes. The mixture was agitated on an orbital platform for an additional 24 hr to obtain a solid, uniform hydrogel. The hydrogel was then moved to a 37°C incubator and stored overnight to allow solvent evaporation and thus provide a flexible, hydratable HA hydrogel film. The resulting film was cut into 1 x 2 cm^2 pieces, sterilized with ethylene oxide gas and stored separately.

(4) TGF-β Release

The release of TGF-β was observed using an *in vivo* mouse model. A 2-cm midline laparotomy was created on a balb/c mouse. The incision was then closed with three sutures spaced 1-cm apart. Next, a GAG-film with TGF-β or a GAG-film without TGF-β was placed over the wound. The skin incision was closed with running sutures. All films were swollen with normal saline before use. An additional control group consisted of laparotomies covered with no film at all. There were a total of 6 mice per group. After a week, the wounds were exposed and evaluated. Defect in lengths in between sutures were measured and histological samples were taken for evaluation.

b) Results

(1) Preparation of HA film with bFGF

LMWHA-ADH was crosslinked with PEG-diald, thereby physically trapping the bFGF in the hydrogel. The hydrogel began to form within 5 minutes after mixing of the HA-ADH and PEG-diald solutions, but was agitated for 24 hrs to assure the formation of a solid, uniform hydrogel. Evaporation of the solvent provided flexible, hydratable GAG hydrogel film. The HA-ADH films used in these investigations were 50% crosslinked (aldehyde:ADH ratio of 1:1 and 0.5:1).

(2) bFGF release

An *in vitro* release assay was used to monitor the release of bFGF from the HA films. The amount of bFGF released was quantified using an ELISA. The results from the assay are shown in Figure 9 and Figure 10. After 72 hrs, approximately 1000 pg of bFGF was released and detected from both the sterilized and unsterilized films. Such results indicate that the *in situ* polymerization method does not appear to harm the antibody epitopes of the growth factor, suggesting it does not harm the growth factor structure or function. Additionally, the process of gas sterilization did not alter the results, indicating that it might be a valid technique for sterilizing the films.

(3) Preparation of GAG film with TGF-β

LMWHA-ADH and CS-ADH were separately crosslinked with PEG-diald, thereby physically trapping TGF-β in the hydrogel. Hydrogels began to form within 5 minutes after mixing of the GAG-ADH and PEG-diald solutions, but was agitated for 24 hrs. to assure the formation of a solid, uniform hydrogel. Evaporation of the solvent provided flexible, hydratable GAG hydrogel film. Both the GAG-ADH films used were 50% crosslinked (aldehyde:ADH ratio of 1:1 and 0.5:1).

(4) TGF-β Release

The release of TGF-β was observed using an *in vivo* mouse model. Gross evaluation of the wounds indicated that those films that released TGF-β, had considerably less defects than those not releasing the growth factor. Additionally, those receiving no film at all had the largest defects; some exhibiting hernias. All the films used in this Example were sterilized with ethylene oxide gas, yet the TGF-β was still active. This confirms the earlier results suggesting that gas sterilization can be used to sterilize GAG-ADH films with growth factors.

11.Example 11. HA oxidation

1 g HA was dissolved in 100 ml H_2O at 37°C by shaking at 150 rpm overnight. 1.07 g $NaIO_4$ (2 equivalent to HA units) was added into the solution, continued shaking at 37°C 190 rpm for 2h. Extensively dialyzed against H_2O for 3 days, lyophilized to give oxidized HA. The oxidation was detected by ^1H NMR of hydrolyzed aldehyde protons (δ = 5.0-5.5 ppm) with the methyl resonance (δ = 1.95 - 2.00 ppm) of the acetamido moiety of the GlcNAc residues of HA as an internal standard.

a) HA hydrogel preparation with HA-ADH crosslinked by oxidized HA

Similar to the crosslinking process of HA-ADH with PEG-dialdehyde, HA-ADH was dissolved in H_2O at a concentration of 5 mg/mL (Solution A). Oxidized HA was dissolved in H_2O at a concentration of 10 mg/mL (Solution B). Volumes of Solutions A and B were added to a small Petri dish to give various molar equivalents of aldehyde and hydrazide functionalities, and the solutions were mixed with gentle swirling. A hydrogel began to form within 60 sec. The mixture was agitated on an orbital platform for an additional 24 hr to obtain a solid, uniform hydrogel.

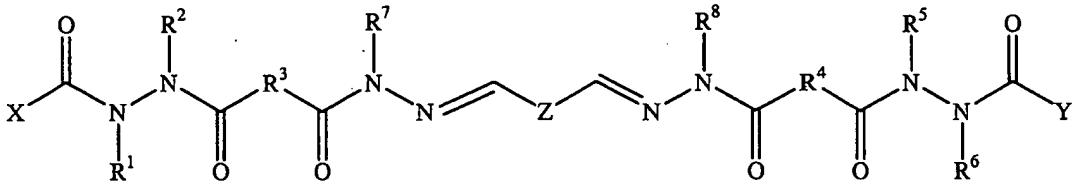
Hydrogels were stored in an open dish overnight at 37 °C to allow solvent evaporation and thus provide a flexible, hydratable HA hydrogel film. Hydrogel films were successfully obtained with the molar ratio of aldehyde/hydrazide equal to 0.05, 0.1, 0.2, 0.5, or 1.0. Similar oxidation will occur with other polysaccharides, e.g. dextran, pectin, cellulose etc. The oxidized polysaccharides could be used as crosslinkers to prepare HA hydrogels. See Figures 11 and 12.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

It will be apparent to those skilled in the art that various modifications and variations can be made in the present invention without departing from the scope or spirit of the invention. Other embodiments of the invention will be apparent to those skilled in the art from consideration of the specification and practice of the invention disclosed herein. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit of the invention being indicated by the following claims.

What is claimed is:

1. A hydrogel film comprising a polymer, wherein the polymer has at least one unit having the formula I



I

wherein

X and Y are a polysaccharide residue; and

Z, R^1, R^2, R^3, R^4, R^5, R^6, R^7, and R^8 are, independently, hydrogen, a polysaccharyl group, a substituted or unsubstituted hydrocarbyl group, a substituted or unsubstituted heterohydrocarbyl group, or a polyether group, wherein Z, R^3, and R^4 are not hydrogen.

2. The film of Claim 1, wherein X and Y are the same polysaccharide residue.

3. The film of Claim 1, wherein X and Y are different polysaccharide residues.

4. The film of Claim 1, wherein the polysaccharide residue is a glycosaminoglycan.

5. The film of Claim 1, wherein X and Y are, independently, a residue of chondroitin sulfate, dermatan, heparan, heparin, dermatan sulfate, heparan sulfate, alginic acid, pectin, or carboxymethylcellulose.

6. The film of Claim 1, wherein X and Y are a residue of hyaluronic acid.

7. The film of Claim 1, wherein Z is a polyether.

8. The film of Claim 1, wherein R^1, R^2, R^5, R^6, R^7, and R^8 are hydrogen.

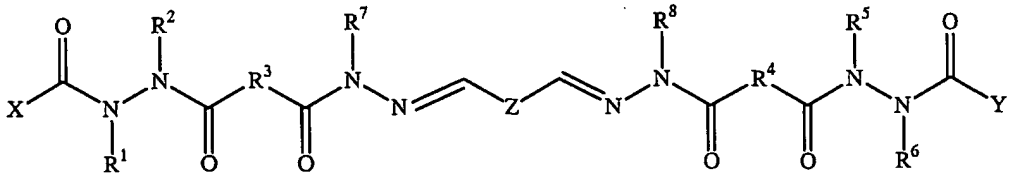
9. The film of Claim 1, wherein R^3 and R^4 are an alkyl group.

10. The film of Claim 1, wherein R^3 and R^4 are $(CH_2)_n$, wherein n is from 1 to 20.

11. The film of Claim 10, wherein n is from 2 to 4.

12. The film of Claim 1, wherein the polymer has from 10 to 10,000 units having the formula I.

13. A hydrogel film comprising a polymer, wherein the polymer has at least one unit having the formula I



I

wherein X and Y are a residue of hyaluronic acid, Z is a polyethylene ether, R^1, R^2, R^5, R^6, R^7, and R^8 are hydrogen, and R^3 and R^4 is $(CH_2)_4$.

14. A hydrogel film comprising a compound having the formula II:

$$[A_x\text{-}B_y\text{-}A_x]_j \quad \text{II}$$

wherein

A is a glycosaminoglycan having at least one hydrazide group;

B is a dialdehyde crosslinker;

x is the number of glycosaminoglycan molecules, which is a whole number in a range of 1 to 100 molecules;

y is the number of dialdehyde crosslinker molecules, which is a whole number in the range of 1 to 10 molecules; and

j is the number of crosslinked glycosaminoglycan-dialdehyde crosslinker- glycosaminoglycan units, which is a whole number in the range of 10 units to 100 million units.

15. A hydrogel film produced by the process comprising reacting (1) a modified polysaccharide having at least one hydrazide group with (2) a polyaldehyde.

16. The film of Claim 15, wherein the modified polysaccharide comprises the reaction product between a polysaccharide having at least one carboxylic acid group and a dihydrazide compound having the formula III

$$H_2N\text{-}NH\text{-}C(O)\text{-}E\text{-}C(O)\text{-}NH\text{-}NH_2 \quad \text{III}$$

wherein E is a substituted or unsubstituted hydrocarbyl group, a substituted or unsubstituted heterohydrocarbyl group, or a polysaccharyl group.

17. The film of Claim 16, wherein the dihydrazide comprises adipic dihydrazide, butandioic dihydrazide, or suberic dihydrazide.

18. The film of Claim 16, wherein the modified polysaccharide comprises the reaction product between hyaluronic acid and adipic dihydrazide.

19. The film of Claim 16, wherein the polyaldehyde is a dialdehyde.

20. The film of Claim 16, wherein the polyadlehyde comprises a polyether dialdehyde.

21. The film of Claim 16, wherein the polyaldehyde is poly(ethylene glycol)-propiondialdehyde.

22. The film of Claim 16, wherein the modified polysaccharide is the reaction product between hyaluronic acid and adipic dihydrazide and the polyaldehyde is poly(ethylene glycol)-propiondialdehyde.

23. A method for producing the hydrogel film of Claim 1, comprising reacting (1) a modified polysaccharide having at least one hydrazide group with (2) a polyaldehyde.

24. A pharmaceutical composition comprising a pharmaceutically-acceptable compound and the hydrogel film of Claim 1.

25. The composition of Claim 24, wherein the pharmaceutically-acceptable compound comprises an antiinflammatory, an antipyretic, a hormone, a growth factor, a contraceptive agent, an analgesic, a hypnotic, a sedative, an anticonvulsant, a muscle relaxant, a local anesthetic, an antispasmodic, an antiulcer drug, a peptidic agonist, a sympathiomimetic agent, a cardiovascular agent, an antitumor agent, an oligonucleotide, a spermicide,

an antibacterial, antineoplastic agent, an antihistamine, an antiviral, an antifungal, an antiproliferative, a vaccine, or a combination thereof.

26. The composition of Claim 24, wherein the pharmaceutically-acceptable compound is a steroid.

27. The composition of Claim 24, wherein the pharmaceutically-acceptable compound comprises pilocarpine, hydrocortisone, prednilosone, cortisone, diclofenac sodium, indomethacin, 6∝-methyl-prednisolone, corticosterone, dexamethasone, prednisone, penicillin, cephalosporins, bacitracin, tetracycline, doxycycline, gentamycin, chloroquine, vidarabine, salicylic acid, acetaminophen, ibuprofen, naproxen, piroxicam, flurbiprofen, morphine, cocaine, lidocaine, benzocaine, fibronectin, human growth hormone, a colony stimulating factor, bone morphogenic protein, platelet-derived growth factor (PDGF), insulin-derived growth factor, transforming growth factor-alpha, transforming growth factor-beta, epidermal growth factor, fibroblast growth factor, interleukin-1, dried bone material, methotrexate, 5-fluorouracil, adriamycin, vinblastine, cisplatin, tumor-specific antibodies conjugated to toxins, tumor necrosis factor, progesterone, testosterone, follicle stimulating hormone, insulin, diphenhydramine, papaverine, streptokinase, isopropamide iodide, metaproternal sulfate, aminophylline, theophylline, niacin, minoxidil, B-adrenergic blocking agent, dopamine, risperidone, naltrexone, naloxone, or buprenorphine.

28. The composition of Claim 24, wherein the composition further comprises an aqueous vehicle, a non-aqueous vehicle, a viscosity enhancing agent, an additive, a buffer, or a combination thereof.

29. A method for producing the pharmaceutical composition, comprising

admixing a pharmaceutically-acceptable compound with the hydrogel film of Claim 1.

30. The method of Claim 29, wherein during the admixing step, the pharmaceutically-acceptable compound reacts with the hydrogel film.

31. A method for producing a pharmaceutical composition, comprising

(a) admixing a pharmaceutically-acceptable compound with a modified polysaccharide having at least one hydrazide group, and

(b) reacting the modified polysaccharide in the admixture of step (a) with a polyaldehyde.

32. The method of Claim 31, wherein during the admixing step (a), the pharmaceutically-acceptable compound reacts with the modified polysaccharide.

33. A method for improving wound healing in a mammal in need of such improvement, comprising contacting the wound of a mammal with the hydrogel film of Claim 1.

34. A method for delivering at least one pharmaceutically-acceptable compound to a patient in need of such delivery, comprising contacting at least one tissue capable of receiving the pharmaceutically-acceptable compound with the composition of Claim 24.

35. The method of Claim 34, wherein the delivery is for a medical purpose comprising the delivery of a contraceptive agent, treating a postsurgical

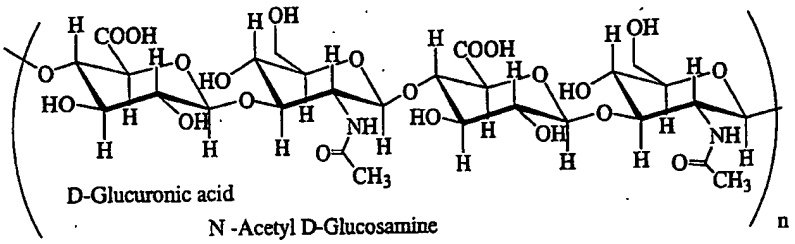
adhesion, promoting skin growth, preventing scarring, dressing a wound, conducting viscosurgery, conducting viscosupplementation, or engineering tissue.

36. A method for purifying a modified polysaccharide having at least one hydrazide group, comprising performing a first dialysis step comprising dialyzing the modified polysaccharide in the presence of a salt.

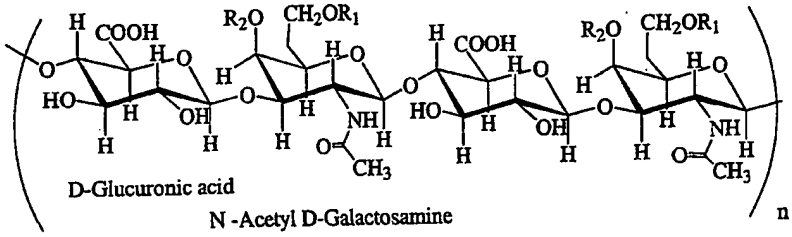
37. The method of Claim 36, wherein the salt is NaCl.

38. The method of Claim 36, wherein after the first dialysis step, performing a second dialysis step comprising dializing the modified polysaccharide in the presence of an aqueous alcohol.

39. The method of Claim 38, wherein after the second dialysis step, performing a third dialysis step comprising dialyzing the modified polysaccharide in the presence of water.



Hyaluronic Acid



Chondroitin 4-sulfate (R_1 = H, R_2 = SO_3^-)

Chondroitin 6-sulfate (R_1 = SO_3^-, R_2 = H)

FIG. 1A

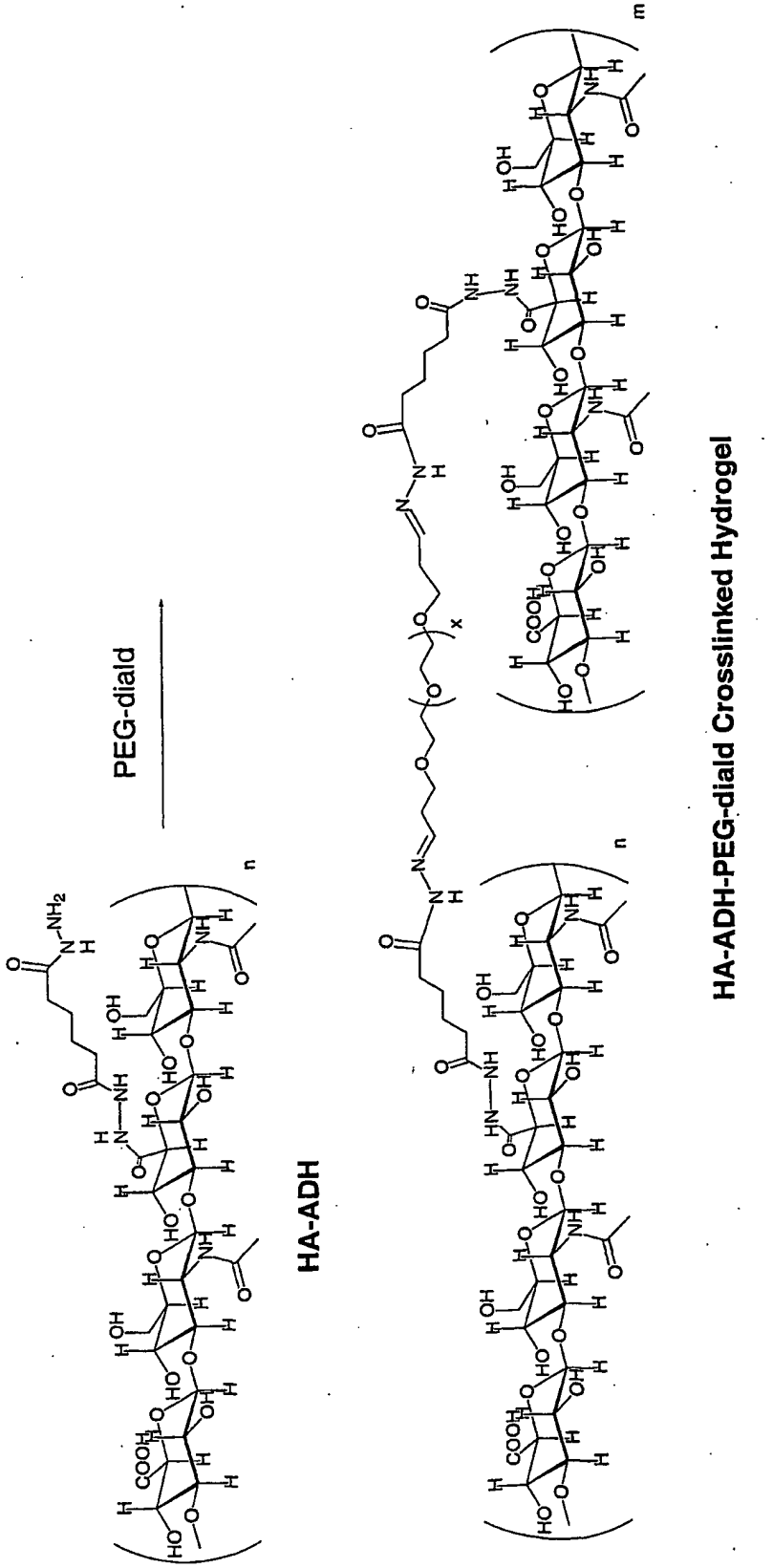


FIG. 1B

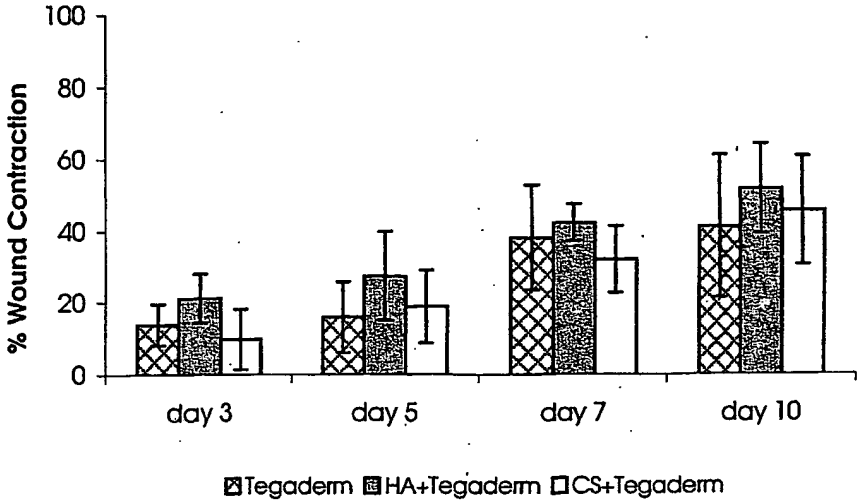

100
80
60
40
20
0
% Wound Contraction
day 3
day 5
day 7
day 10
Tegaderm
HA+Tegaderm
CS+Tegaderm

FIG. 2

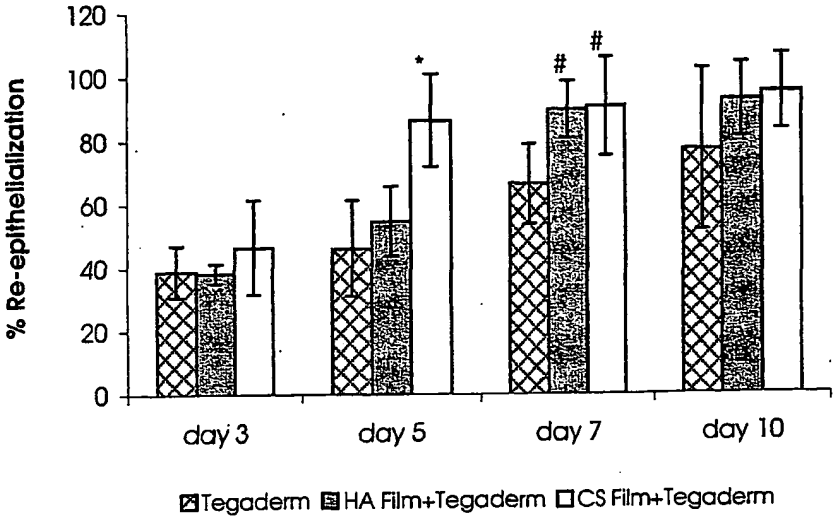

% Re-epithelialization
120
100
80
60
40
20
0
*
#
#
day 3
day 5
day 7
day 10
Tegaderm
HA Film+Tegaderm
CS Film+Tegaderm

FIG. 3

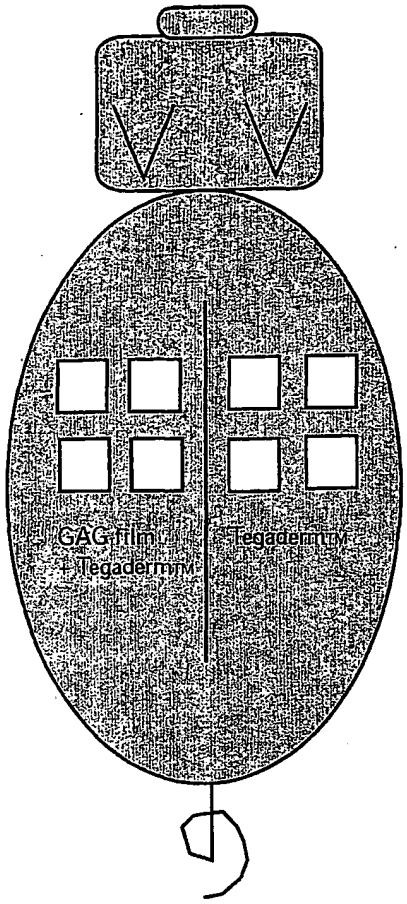
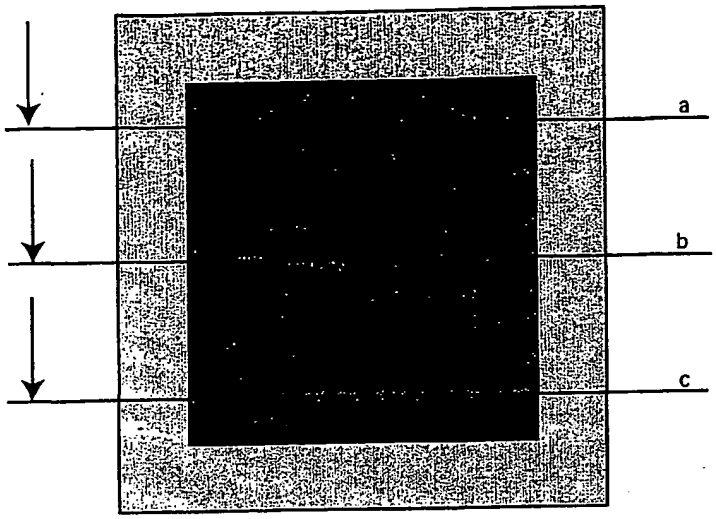


FIG. 4



(a)

(b)

FIG. 5

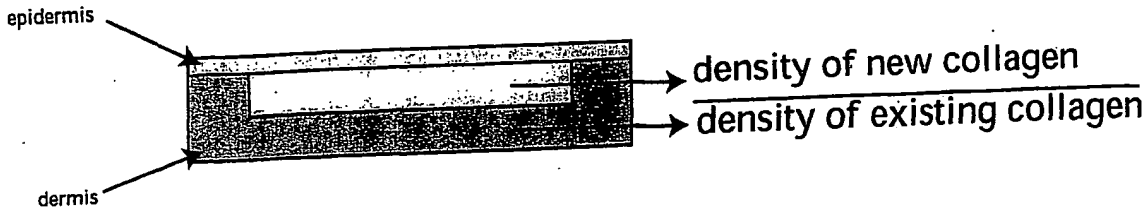

epidermis
dermis
density of new collagen
density of existing collagen

FIG. 6

% covered	wound 1	wound 2	wound 3	wound 4	average	stdev
Tegaderm®	28.8758867	15.182063	59.2948718	33.7166667	34.267372	18.4390542
HA+Tegader	72.5786164	64.5700411	48.1168494	62.2108844	61.8690978	10.1854477

% covered	wound 1	wound 2	wound 3	wound 4	average	stdev
Tegaderm®	100	100	100	100	100	0
HA+Tegader	83.9285849	100	100	100	95.9821462	8.03570753

	day 3	day 7	stdev 3	stdev 7
Tegaderm	34.267372	100	18.4390542	0
HA film+Tegaderm	61.8690978	95.9821462	10.1854477	8.03570753

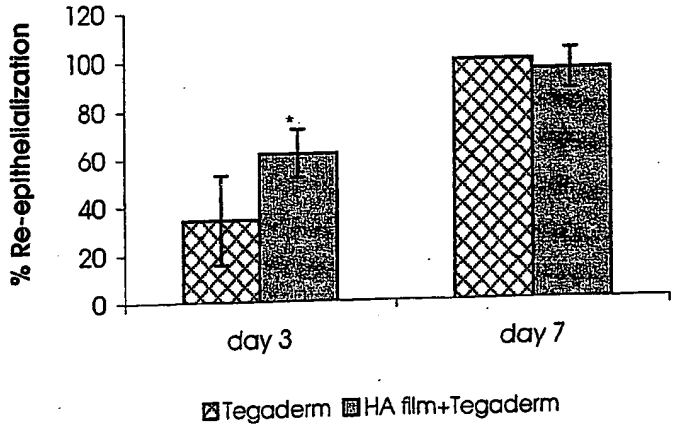
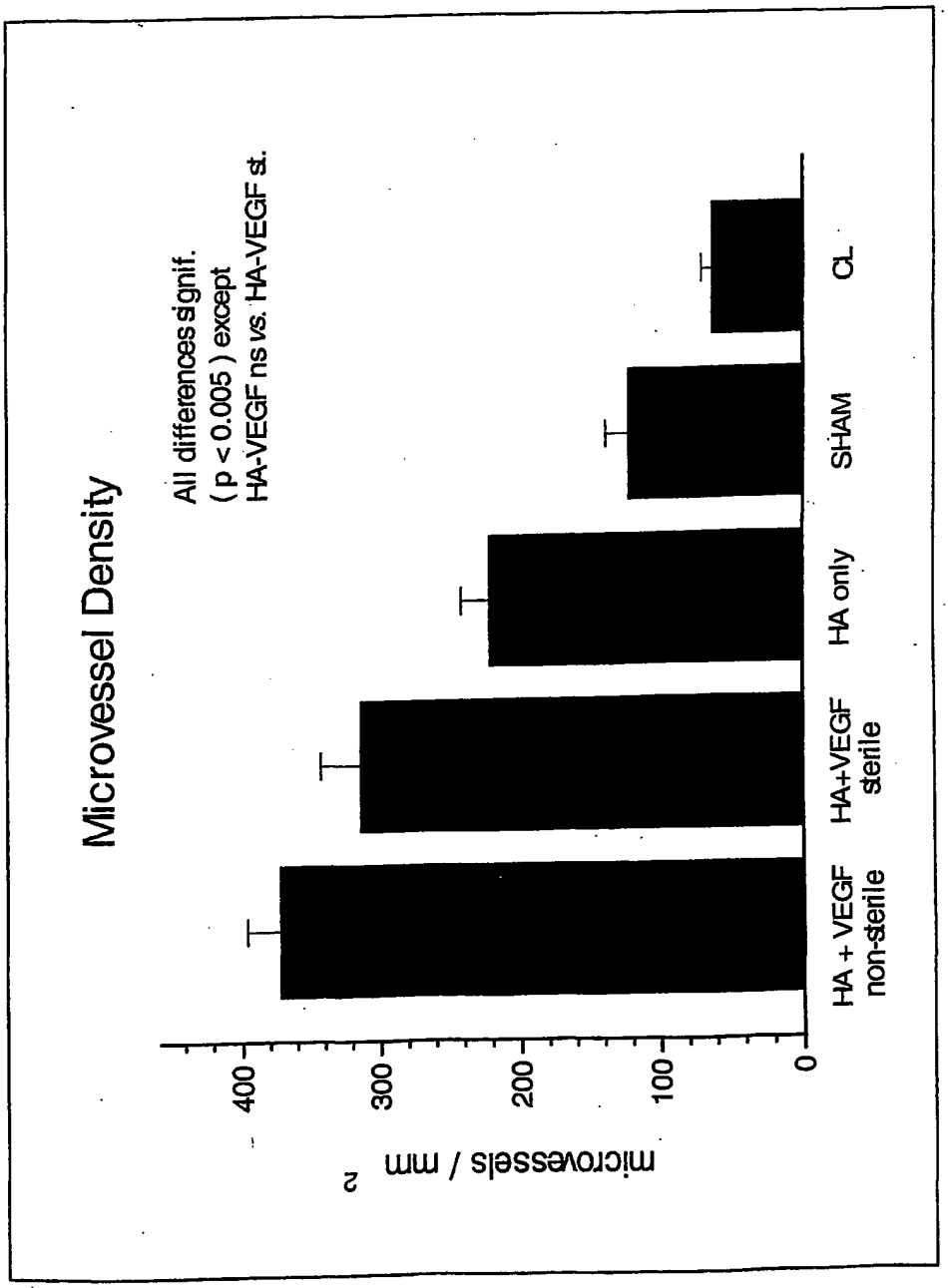


FIG. 7

FIG. 8


Microvessel Density
All differences signif.
(p < 0.005) except
HA-VEGF ns vs. HA-VEGF st.
microvessels / mm 2
0
100
200
300
400
HA + VEGF non-sterile
HA+VEGF sterile
HA only
SHAM
CL

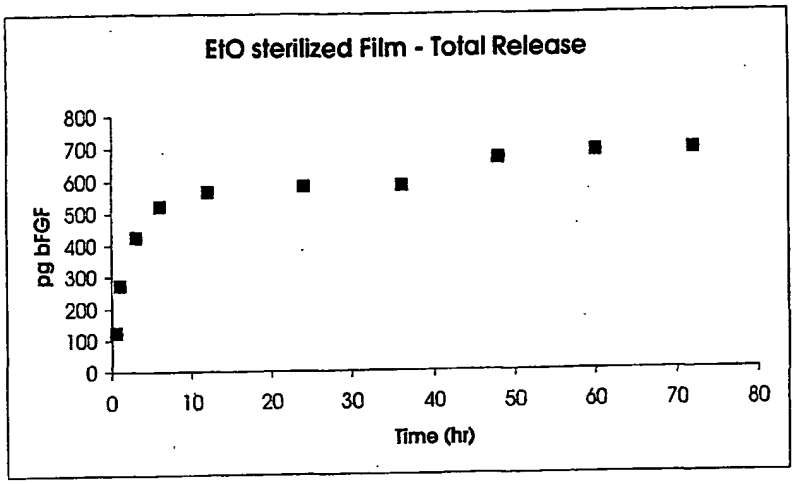

EtO sterilized Film - Total Release
pg bFGF
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
80
Time (hr)

FIG. 9

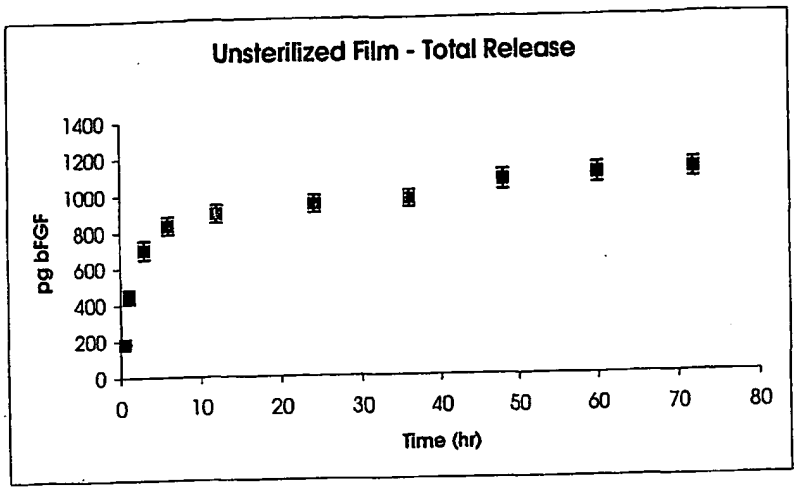

Unsterilized Film - Total Release
pg bFGF
1400
1200
1000
800
600
400
200
0
0
10
20
30
40
50
60
70
80
Time (hr)

FIG. 10

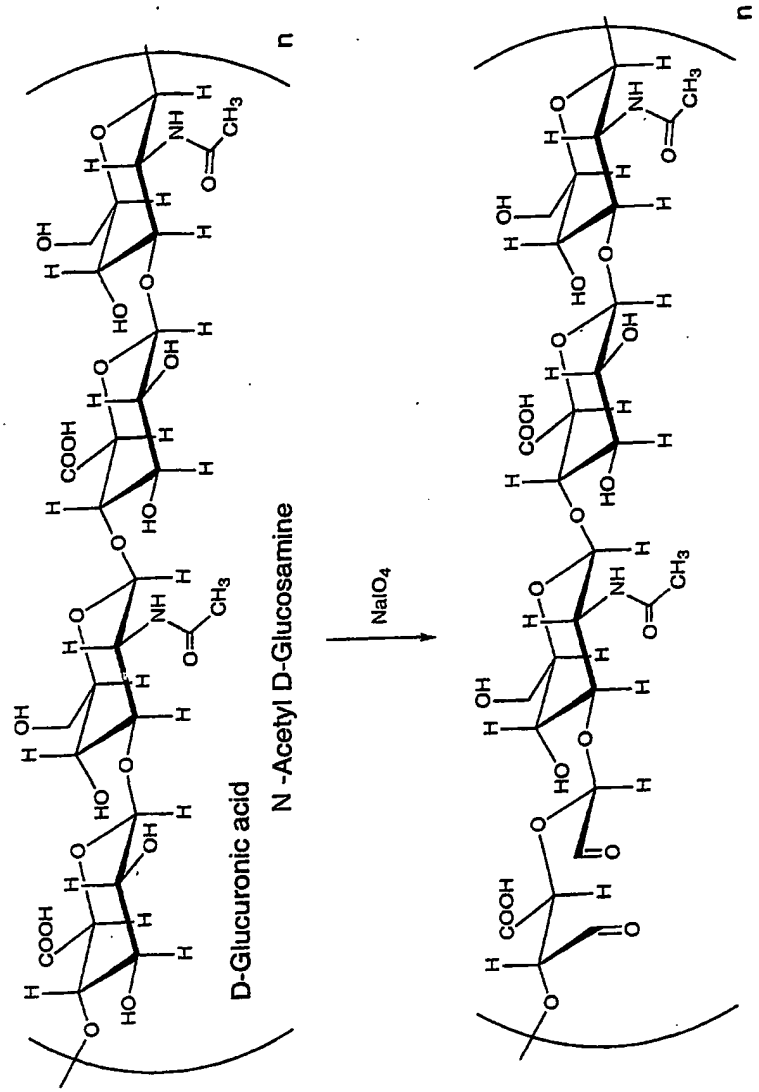


FIG. 11

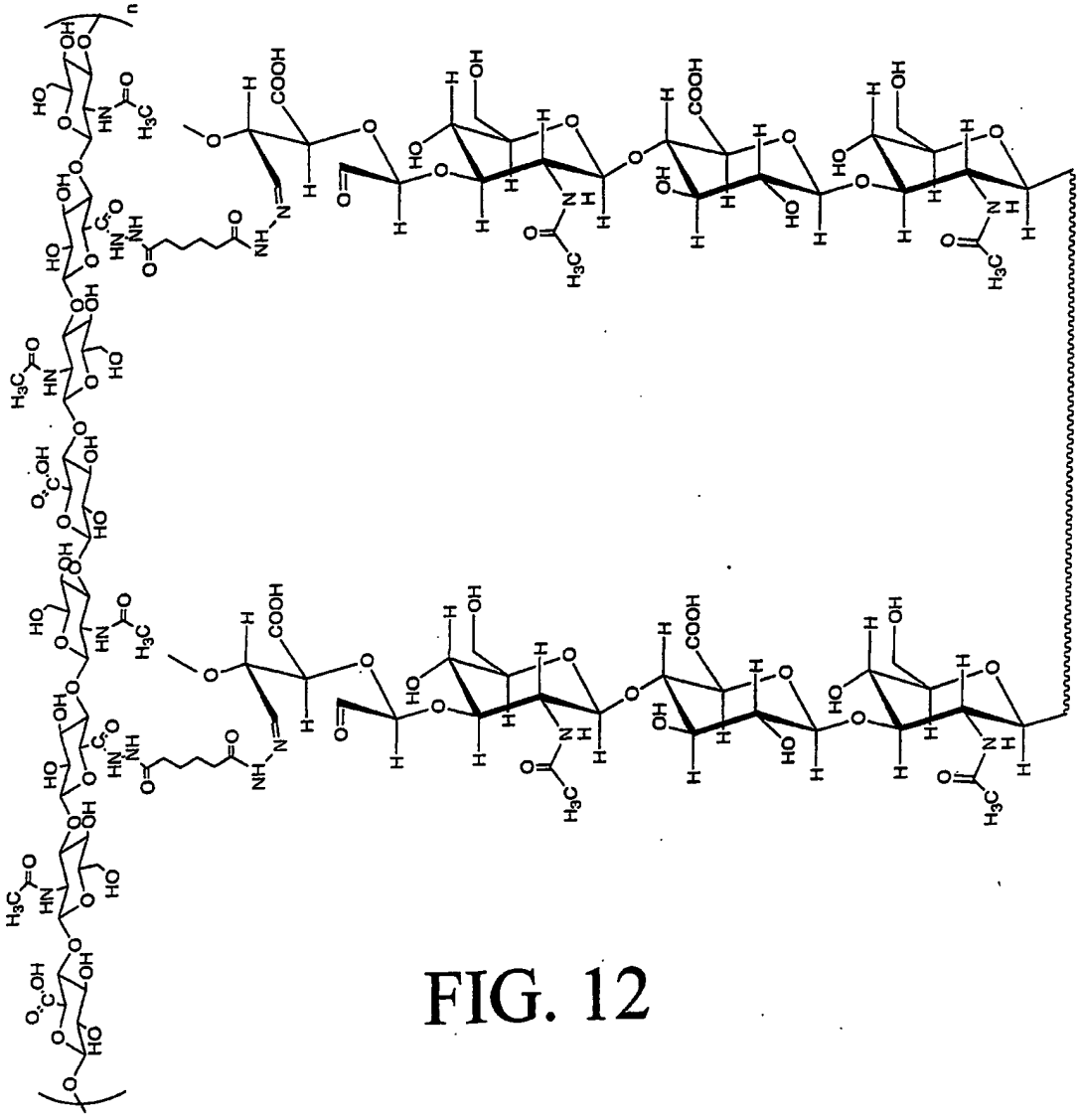


FIG. 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/22556

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C08G 63/48, 63/91; A61K 9/14

US CL :525/54.2,54.21,54.23,54.3; 424/484,486,488

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : C08G 63/48, 63/91; A61K 9/14

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,242,828 A (BERGSTROM et al) 07 September 1993, see entire document.	1-39
A	US 5,492,840 A (MALMQVIST et al) 20 February 1996, see entire document.	1-39
A, P	US 6,180,288 B1 (EVERHART et al) 30 January 2001, see entire document.	1-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
26 SEPTEMBER 2001	10 OCT 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NATHAN M. NUTTER Jean Proctor, Paralegal Specialist Telephone No. (703) 308-0661

Form PCT/ISA/210 (second sheet) (July 1998)★